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Pathways of dissemination in colorectal cancer

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Colofon

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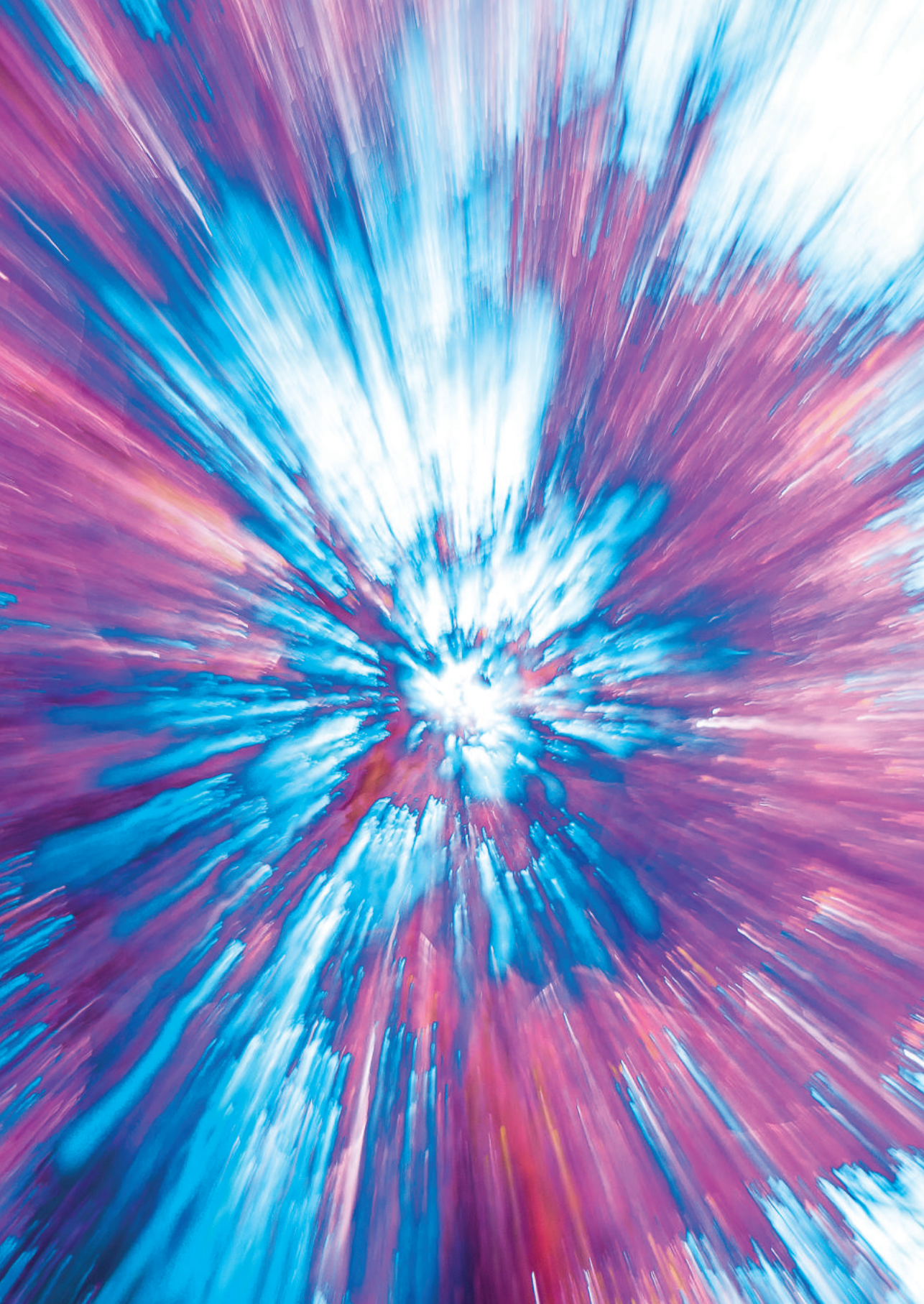


General introduction and outline of the thesis

Colorectal cancer

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in the western world and accounts for many cancer related deaths.[1] The life-time risk of developing CRC is around 5%.[2] Treatment of primary CRC is based on surgery, complemented either with neoadjuvant therapy mainly for rectal cancer, and – dependent on the stage of disease – adjuvant systemic treatment mainly for colon cancer. Metastatic disease is the leading factor in CRC mortality. Currently, approximately 20% of CRC patients have metastatic disease at time of first presentation[3, 4] and another 20-30% of patients will develop distant metastases during follow-up[5-7] despite intensive follow-up and increasing therapeutic options for CRC. Population screening for CRC has been implemented since 2014. This is supposed to increase early detection of colorectal cancer and thereby reduce mortality.

Staging

Disease management is essentially based on tumor stage. Staging of surgical resection specimens by pathology is considered the most accurate determination of local extent of disease, and as such the most powerful and reliable predictor of prognosis of primary CRC[8, 9]. Therefore, staging plays an essential role in choosing the most appropriate therapy for CRC patients.

In 1932 Cuthbert Dukes proposed a classification for rectal cancer, based on the extent of disease evaluated by the degree of tumor infiltration through the bowel wall and the presence or absence of lymph node metastases.[10] (Figure 1) The Dukes classification underwent several modifications by Dukes and other investigators, but still forms the backbone of the current staging system for CRC; the TNM classification. This classification is based on the assessment of the anatomic extent of disease at the time of diagnosis with a key role for lymph node metastases.[11]

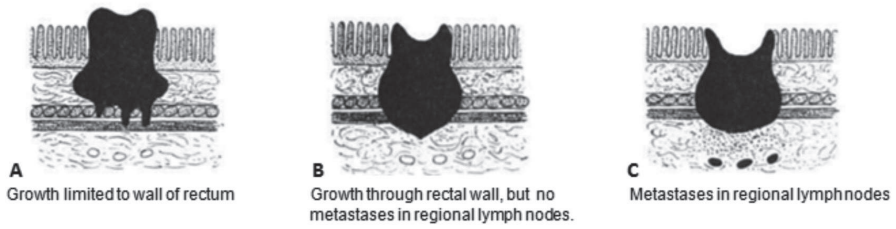


Figure 1. Adapted from Dukes et al.[10]

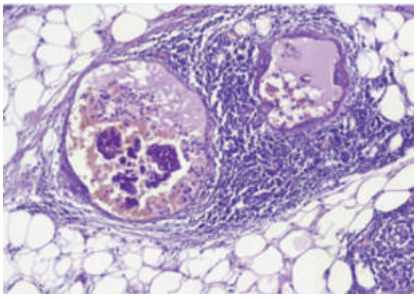
The three pillars of the TNM system are: **T** stage, based on the depth of tumor infiltration, **N** stage, based on the presence of lymph node metastases, **M** stage, based on the presence of distant metastases. The number of categories in the TNM classification is expanded compared to original Dukes staging, with more categories of tumor invasion depth, with separate categories based on the number of regional lymph node metastases (stage III) and the inclusion of distant metastases (stage IV). Adjuvant chemotherapy is recommended for all patients with stage III colon cancer and in selected high risk stage II patients: pT4, presentation with perforation or obstruction, less than 10 lymph nodes examined, and/or vascular invasion.[12] The 2017 update of the Dutch guideline for medical oncology has restricted these factors to pT4 stage.

Histology

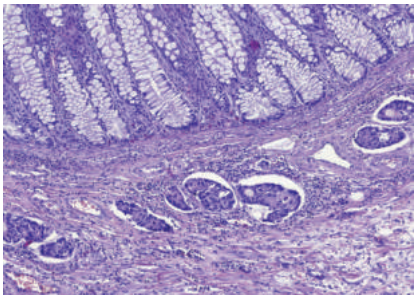
In addition to staging, other important histological factors in CRC are tumor type, tumor grade, resection margins, venous invasion and lymphatic invasion. CRC can be subdivided into different tumor types. The majority of CRCs are conventional adenocarcinomas, other tumor types include mucinous adenocarcinoma, signet-ring cell, medullary, micropapillary, serrated, adenosquamous, spindle cell and undifferentiated carcinoma, some of which are extremely rare. Conventional adenocarcinomas are graded according to the degree of gland formation. Good differentiation shows more than 95% gland formation, moderate differentiation shows 50-95% gland formation and poor differentiation shows less than 50% gland formation. A 2-tiered grading system, which combines well and moderately differentiated versus poorly differentiated, reduces interobserver variation and improves prognostic significance.[13] Although controversial, tumor grade is generally considered as a stage-independent prognostic variable, and poorly differentiated histology is associated with poor patient survival.

Venous invasion is the presence of tumor cells within venous blood vessels (Figure 2a). It can be subdivided into intramural venous invasion (IMVI) and extramural venous invasion (EMVI), according to the location of the vessels towards the muscle wall. EMVI is a well-established independent prognostic indicator, associated with hematogenous spread and mortality in CRC. The importance of EMVI is recognized by the UK Royal College of Pathologists, which has adjusted its minimum audit standard of EMVI detection to 30% in CRC resections.[14] Next to EMVI, there is now increasing evidence that IMVI may also be of prognostic importance, although to a lesser extent.[15-17] Lymphatic invasion is the presence of tumor cells within lymphatic vessels (Figure 2b). The evidence for lymphatic invasion as a prognostic factor in colorectal cancer resection specimens is limited and it is considered a non-core dataset item for resection specimens.[14] However for superficial tumors (T1), lymphatic invasion is regarded as a significant risk factor for lymph node metastases.[18] Perineural invasion

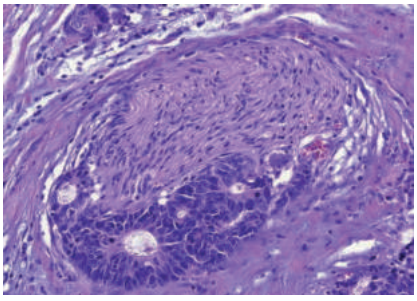
(PNI) is the growth of tumor cells along or within nerves (Figure 2c). PNI is observed in up to 33% of colorectal tumors and is associated with a worse prognosis in some studies.[19] It is included as an accessory feature in pathology reports, because its prognostic value in CRC is not validated. In many CRC resection specimens nodules or foci of cancer cells can be found in the pericolic or perirectal fat, without evidence of residual lymph node tissue. Those nodules are called tumor deposits (Figure 2d). Tumor deposits first appeared in the fifth edition of the TNM staging system.[20, 21] In TNM5 tumor deposits are considered as lymph node metastases if they have a diameter greater than 3 mm, and tumor deposits smaller than 3 mm are classified in T category as discontinuous extension. In TNM6 the 3-mm rule is withdrawn and replaced by a definition of tumor deposits based on contour.[22, 23] Tumor deposits are classified as lymph node metastases when they have the form and smooth contour of lymph nodes; irregular tumor deposits are classified in the T category and as venous invasion. In TNM7 and TNM8, classification of tumor deposits is left to the opinion of the pathologist, and a new N category (N1c) has been designed to include patients with tumor deposits in stage III.[11, 24] There are different opinions about the origin of tumor deposits, such as being totally replaced lymph nodes, vascular invasion or a direct extension of the primary tumor. The prognostic role of tumor deposits in CRC has still to be elucidated.



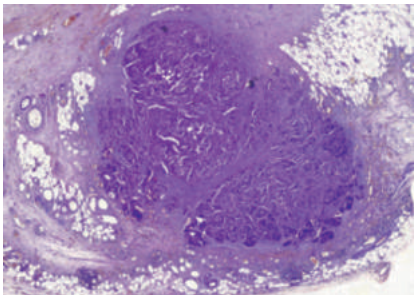
2a. venous invasion



2b. lymphatic invasion



2c. perineural invasion



2d. tumor deposit

Figure 2.

Metastases

A key feature of cancer cells is their ability to dissociate from the primary tumor and form metastatic deposits. Colorectal tumors can move to other parts of the body using different pre-existing pathways, which can be investigated under the microscope. As mentioned above, tumor cells can invade blood vessels (venous invasion), lymphatic vessels and lymph nodes (lymph node invasion), grow along nerves (perineural invasion) or directly grow through the bowel wall (Figure 3). This direct growth of the tumor through the bowel wall is considered the key element for dissemination within the peritoneal cavity (peritoneal carcinomatosis). Perineural invasion may be important in local spread, while vascular and lymphatic invasion may be more important in dissemination to distant organs. Although staging and treatment of CRC is primarily based on regional lymph node metastases, other factors like vascular invasion, may be more direct markers of dissemination to distant organs. Approximately 40% of patients who develop liver metastases after curative resection of the primary tumor do not have regional lymph node metastases upon diagnosis.[25] Therefore it is important to investigate the role of other factors, like vascular invasion and tumor deposits, in dissemination of CRC. Next to histological factors, the genetic background of colorectal tumors could explain different patterns of dissemination.

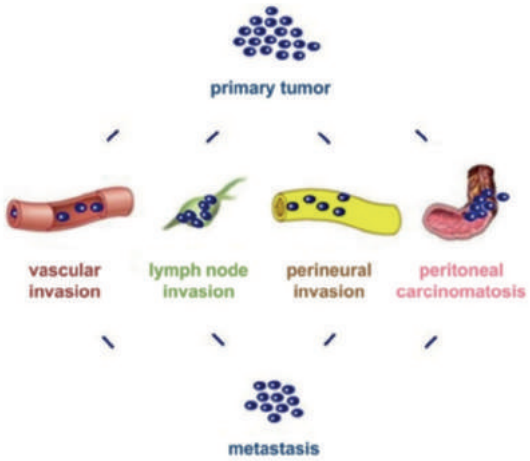


Figure 3.

Somatic mutations have been linked to prognosis as well as to patterns of dissemination of CRC.[26] *(K)RAS* and *BRAFV600E* mutations indicate a worse prognosis compared to *RAS/BRAF* wildtype tumors.[27] In metastatic disease, the worse prognosis of tumors with a deficient mismatch repair (dMMR) system is mainly driven by *BRAF* mutation

status.[28] *KRAS* mutations are associated with metastases outside the liver, particularly in the lung, but also in the brain and bone[26, 29] and *BRAF* mutations are associated with decreased liver-limited metastasis and increased peritoneal and lymph node metastases.[26, 30] In 2015 four consensus molecular subtypes (CMS) have been described for CRC, each with distinguishing features (CMS1: microsatellite unstable, hypermutated, strong immune activation, CMS2: epithelial with marked WNT and MYC signaling activation, CMS3: epithelial with metabolic dysregulation and CMS4: mesenchymal, stromal invasion and angiogenesis).[31] Since, for instance, *BRAFV600E* mutations are not restricted to a single CMS, this shows that the prognosis depends on many factors and not just on a single molecular marker. Another promising area is the detection of circulating tumor DNA (ctDNA), which may early identify patients at high risk for the development of metastases.[32]

Treatment of metastatic disease

Approximately half of the CRC patients will develop distant metastases. A minority of patients are candidates for immediate surgery of (mostly liver) metastases, sometimes preceded by induction systemic treatment to achieve downsizing of metastases, with curative intent. Surgery of metastases is increasingly preformed in patients with colorectal liver metastases and in patients with lung metastases[33] due to improvements in systemic regimens, imaging, surgical techniques and perioperative care.[34, 35]

The remainder of patients may be treated with palliative systemic treatment with prolongation of life and maintaining quality of life as major goals. Backbones of systemic treatment are cytotoxic agents such as the fluoropyrimidines, irinotecan and oxaliplatin. Another class of agents, the so-called "targeted therapy" has added further benefit.[36] Three monoclonal antibodies have been approved for clinical use in CRC: bevacizumab, an antibody against the vascular endothelial growth factor (VEGF), and cetuximab and panitumumab, antibodies against the epidermal growth factor receptor (EGFR). Response to anti-EGFR treatment is restricted to patients without mutations in the proto-oncogenes *KRAS NRAS* and *BRAF*.[37, 38] Mutations in *RAS* are observed in 53% of colorectal tumors.[38] These proteins are part of the RAS/RAF/MAPK signal transduction cascade, where an activating mutation leads to constitutive activation of this pathway. In case of these activating mutations, inhibition of the pathway proximal to RAS will have no effect. While colorectal cancer was considered in the past as a single entity, it now has become clear that it is a very heterogeneous disease with many different subgroups that require different treatment approaches.[39]

The 5-year overall survival for patients who are diagnosed with distant metastases is currently around 20%[40] and is, amongst others, strongly correlated with the location of metastatic disease and the possibility for surgical resection of metastases with curative intent.

Metastatic patterns

The most common site of distant metastases from colorectal cancer is the liver, followed by metastases in lung and peritoneal cavity.[33, 41] Next to the liver, lung and peritoneum, various other metastatic sites such as bone, spleen, brain and distant lymph nodes have been described.[42-44] Although differences in metastatic locations of various types of cancers, but also within a type of cancer, have been noticed, the precise biological mechanisms by which individual tumors disseminate to secondary sites remain unknown. Two hypotheses attempt to explain differences in metastatic patterns; the mechanistical hypothesis[45] and the seed-and-soil hypothesis.[46] According to the mechanistical view of metastatic spread, tumor cells can disseminate to distant organs through two pathways: the vascular and the lymphatic pathway. In the vascular pathway blood vessels transport tumor cells directly to distant organs. Hematogenous routes of CRC metastases run via the portal vein (involved in liver metastases), the systemic veins (involved in lung metastases), arteries (leading to metastatic deposits in all organs) or the venous plexus of Batson (leading to bone metastases). In the lymphatic pathway tumor cells may disseminate from regional lymph nodes to distant lymph nodes, reach the systemic circulation and subsequently form organ metastases.[47] As mentioned before, both vascular invasion and lymphatic invasion can be evaluated under de microscope and are associated with poor prognosis. However, the presence of tumor cells within a blood or lymph vessels only demonstrates the capability of tumor cells to invade other structures. It does not necessarily demonstrate the ability of the tumor cells to survive in blood vessels and their capability to form metastasis at another site with a different microenvironment. The second hypothesis, the seed-and soil hypothesis, is partly based on this assumption. According to the seed-and-soil hypothesis the distribution of distant metastases from a primary tumor is compared with the seeding of a plant: its seeds are carried in all directions, but they can live and grow only if they fall on congenial soil. In this hypothesis each 'host' organ is supposed to have its own microenvironment and each metastasis its own preferences.

Outline of the thesis

Liver metastases are the most common metastases in CRC. Therefore, the first part of the thesis is based on research on colorectal liver metastases. In the first part of this thesis, both histological and molecular factors in colorectal liver metastases are investigated. Compared to the extensive research that is already performed on histological factors in primary CRC, less is known about histological factors in colorectal liver metastases. In **chapter 1** we review the literature regarding histological features in colorectal liver metastases and suggestions are made on factors that should be included in the pathology reports. In **chapter 2** we describe

the impact of intrahepatic dissemination on outcome in patients with colorectal liver metastases. We investigate the role of perineural invasion, lymphatic invasion, and vascular invasion of tumor cells within the liver.

Identification of mutations in the proto-oncogenes *KRAS* and *NRAS* as predictive markers for response to anti-EGFR therapy has improved patient selection, however even in patients with *RAS* wild type tumors the response to anti-EGFR therapy is limited. We investigate whether this suboptimal response can be explained by differences in mutation status between the primary tumor and the metastases. **Chapter 3** presents data on the concordance in *KRAS* mutation status between primary tumors and their corresponding liver metastases. In **chapter 4** we describe our expanded molecular research of primary tumors with corresponding liver and lung metastases. Next to *KRAS* mutation status, mutation status of the proto-oncogenes *BRAF, HRAS, NRAS* and *PIK3CA* are investigated. Mutations in patients with liver metastases and lung metastases are compared, in order to investigate whether convergent evolution explains different patterns of dissemination.

The results on the association of tumor deposits, vascular invasion and lymph node invasion on metastatic patterns are described in **chapter 5**. The prognostic value of tumor deposits in CRC is investigated by systematically reviewing the literature. Moreover, the impact of tumor deposits on metastatic patterns is investigated in four large cohorts and compared with the impact of vascular invasion and lymph node metastases. In **chapter 6** we describe the influence of lymph node metastases on metastatic patterns in CRC. We performed a large autopsy study in which we compare patterns of metastases according to lymph node status and validate our results with a population based study. **Chapter 7** concerns a meta-analysis on the impact of perineural invasion on survival in patients with CRC. A meta-analysis on the impact of intramural vascular invasion on survival is described in **chapter 8**. In the **appendix** an item about optimal reporting of research results is included. We developed a guideline to improve the reporting of retrospective histopathology studies, with the intention to facilitate the comparison of results across different studies.

References

1. Torre, L.A., et al., *Global cancer statistics, 2012.* CA Cancer J Clin, 2015. **65**(2): p. 87-108.
2. Weitz, J., et al., *Colorectal cancer.* Lancet, 2005. **365**(9454): p. 153-65.
3. Cook, A.D., R. Single, and L.E. McCahill, *Surgical resection of primary tumors in patients who present with stage IV colorectal cancer: an analysis of surveillance, epidemiology, and end results data, 1988 to 2000.* Ann Surg Oncol, 2005. **12**(8): p. 637-45.
4. Meulenbeld, H.J., et al., *Significant improvement in survival of patients presenting with metastatic colon cancer in the south of The Netherlands from 1990 to 2004.* Ann Oncol, 2008. **19**(9): p. 1600-4.
5. Guren, M.G., et al., *Nationwide improvement of rectal cancer treatment outcomes in Norway, 1993-2010.* Acta Oncol, 2015: p. 1-9.
6. Manfredi, S., et al., *Incidence and patterns of recurrence after resection for cure of colonic cancer in a well defined population.* Br J Surg, 2006. **93**(9): p. 1115-22.
7. van Gestel, Y.R., et al., *Patterns of metachronous metastases after curative treatment of colorectal cancer.* Cancer Epidemiol, 2014. **38**(4): p. 448-54.
8. Gunderson, L.L., et al., *Revised tumor and node categorization for rectal cancer based on surveillance, epidemiology, and end results and rectal pooled analysis outcomes.* J Clin Oncol, 2010. **28**(2): p. 256-63.
9. Gunderson, L.L., et al., *Revised TN categorization for colon cancer based on national survival outcomes data.* J Clin Oncol, 2010. **28**(2): p. 264-71.
10. Dukes, C.E., *The classification of cancer of the rectum.* The Journal of Pathology and Bacteriology, 1932. **35**(3): p. 323-332.
11. Edge, S.B., *AJCC cancer staging manual*. 7th ed. / edited by Stephen B. Edge ... [et al.]. ed. 2010, New York ; London: Springer.
12. Figueredo, A., M.E. Coombes, and S. Mukherjee, *Adjuvant therapy for completely resected stage II colon cancer.* Cochrane Database Syst Rev, 2008(3): p. CD005390.
13. Fleming, M., et al., *Colorectal carcinoma: Pathologic aspects.* J Gastrointest Oncol, 2012. **3**(3): p. 153-73.
14. Loughrey, M.B.Q., P.;,Shepherd, N. A. . *Dataset for Colorectal Cancer Histopathology Reports.* 2014; Available from: http://www.rcpath.org/Resources/RCPath/Migrated%20Resources/Documents/G/G049_ColorectalDataset_July14.pdf.
15. Betge, J., et al., Intramural and extramural *vascular invasion in colorectal cancer: prognostic significance and quality of pathology reporting.* Cancer, 2012. **118**(3): p. 628-38.
16. Petersen, V.C., et al., *Identification of objective pathological prognostic determinants and models of prognosis in Dukes' B colon cancer.* Gut, 2002. **51**(1): p. 65-9.
17. Roxburgh, C.S., et al., *Elastica staining for venous invasion results in superior prediction of cancer-specific survival in colorectal cancer.* Ann Surg, 2010. **252**(6): p. 989-97.
18. Bosch, S.L., et al., *Predicting lymph node metastasis in pT1 colorectal cancer: a systematic review of risk factors providing rationale for therapy decisions.* Endoscopy, 2013. **45**(10): p. 827-34.
19. Liebig, C., et al., *Perineural invasion in cancer: a review of the literature.* Cancer, 2009. **115**(15): p. 3379-91.
20. *AJCC cancer staging manual*. 5th ed. / American Joint Committee on Cancer ... ed. 1997, Philadelphia: Lippincott-Raven.
21. Sobin, L.H. and C. Wittekind, *TNM classification of malignant tumours*. 5th ed. / edited by L.H. Sobin and Ch. Wittekind. ed. 1997, New York ; Chichester: Wiley.
22. Greene, F.L., *AJCC cancer staging handbook : TNM classification of malignant tumors*. 6th ed. / F.L. Greene ... [et al.] ed. 2002, New York ; London: Springer.
23. Sobin, L.H. and C. Wittekind, *TNM classification of malignant tumours*. 6th ed. / edited by L.H. Sobin and Ch. Wittekind. ed. 2002, New York ; [Chichester]: Wiley-Liss.
24. Sobin, L.H., M.K. Gospodarowicz, and C. Wittekind, *TNM classification of malignant tumours*. 7th ed. ed. 2010, Oxford: Wiley-Blackwell.

25. Mekenkamp, L.J., et al., *Clinicopathological features and outcome in advanced colorectal cancer patients with synchronous vs metachronous metastases.* Br J Cancer, 2010. **103**(2): p. 159-64.
26. Lipsyc, M. and R. Yaeger, *Impact of somatic mutations on patterns of metastasis in colorectal cancer.* Journal of gastrointestinal oncology, 2015. **6**(6): p. 645-9.
27. Cremolini, C., et al., *FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab as first-line treatment of patients with metastatic colorectal cancer: updated overall survival and molecular subgroup analyses of the open-label, phase 3 TRIBE study.* Lancet Oncol, 2015. **16**(13): p. 1306-15.
28. Venderbosch, S., et al., *Mismatch repair status and BRAF mutation status in metastatic colorectal cancer patients: a pooled analysis of the CAIRO, CAIRO2, COIN, and FOCUS studies.* Clin Cancer Res, 2014. **20**(20): p. 5322-30.
29. Cejas, P., et al., *KRAS mutations in primary colorectal cancer tumors and related metastases: a potential role in prediction of lung metastasis.* PLoS One, 2009. **4**(12): p. e8199.
30. Tran, B., et al., *Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer.* Cancer, 2011. **117**(20): p. 4623-32.
31. Guinney, J., et al., *The consensus molecular subtypes of colorectal cancer.* Nature medicine, 2015. **21**(11): p. 1350-6.
32. Phallen, J., et al., *Direct detection of early-stage cancers using circulating tumor DNA.* Sci Transl Med, 2017. **9**(403).
33. van der Geest, L.G., et al., *Nationwide trends in incidence, treatment and survival of colorectal cancer patients with synchronous metastases.* Clin Exp Metastasis, 2015. **32**(5): p. 457-65.
34. Jeong, S., et al., *Surgical resection of synchronous and metachronous lung and liver metastases of colorectal cancers.* Ann Surg Treat Res, 2017. **92**(2): p. 82-89.
35. Poston, G., et al., *The role of cetuximab in converting initially unresectable colorectal cancer liver metastases for resection.* Eur J Surg Oncol, 2017.
36. Knijn, N., J. Tol, and C.J. Punt, *Current issues in the targeted therapy of advanced colorectal cancer.* Discov Med, 2010. **9**(47): p. 328-36.
37. Karapetis, C.S., et al., *K-ras mutations and benefit from cetuximab in advanced colorectal cancer.* N Engl J Med, 2008. **359**(17): p. 1757-65.
38. Sorich, M.J., et al., *Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized, controlled trials.* Ann Oncol, 2015. **26**(1): p. 13-21.
39. Punt, C.J., M. Koopman, and L. Vermeulen, *From tumour heterogeneity to advances in precision treatment of colorectal cancer.* Nat Rev Clin Oncol, 2017. **14**(4): p. 235-246.
40. Kopetz, S., et al., *Improved survival in metastatic colorectal cancer is associated with adoption of hepatic resection and improved chemotherapy.* J Clin Oncol, 2009. **27**(22): p. 3677-83.
41. Riihimaki, M., et al., *Patterns of metastasis in colon and rectal cancer.* Sci Rep, 2016. **6**: p. 29765.
42. Disibio, G. and S.W. French, *Metastatic patterns of cancers: results from a large autopsy study.* Arch Pathol Lab Med, 2008. **132**(6): p. 931-9.
43. Hess, K.R., et al., *Metastatic patterns in adenocarcinoma.* Cancer, 2006. **106**(7): p. 1624-33.
44. Weiss, L., et al., *Haematogenous metastatic patterns in colonic carcinoma: an analysis of 1541 necropsies.* J Pathol, 1986. **150**(3): p. 195-203.
45. Ewing, J., *Neoplastic diseases; a treatise on tumors*. 1928, Philadelphia and London: W. B. Saunders Co.
46. Paget, S., *The distribution of secondary growths in cancer of the breast.* The Lancet, 1889. **133**(3421): p. 571-573.
47. Alitalo, A. and M. Detmar, *Interaction of tumor cells and lymphatic vessels in cancer progression.* Oncogene, 2012. **31**(42): p. 4499-508.

Chapter 1

Histopathological evaluation of resected colorectal cancer liver metastases: what should be done?

N. Knijn, J.A.M. de Ridder, C.J.A. Punt, J.H.W. de Wilt, I.D. Nagtegaal

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Abstract

Histological reporting of hepatic resections of colorectal liver metastases (CRLMs) is limited to confirmation of diagnosis and evaluation of resection margins. More exhaustive diagnostic reporting might be warranted.

Here, we critically and systematically review the potentially important histological prognostic factors in CRLM. Histological features such as intrahepatic spread, resection margins, and tumour response to neoadjuvant chemotherapy have been defined. Intrahepatic spread (venous, lymphatic, bile duct and perineural invasion) was evaluated in a number of studies.

Meta-analysis demonstrated a clear correlation between 5-year overall survival and both portal vein invasion (RR 1.8, 95% CI 1.3–2.5) and lymphatic invasion (RR 1.7, 95% CI 1.4–2.0). The impact of hepatic vein invasion and bile duct invasion on outcome is not clear. Perineural invasion was linked to survival in one study. Resection margin is an important prognostic factor; however, the significance of the width of negative margins remains controversial. Various studies have evaluated tumour response to neoadjuvant chemotherapy, but different grading systems were used, and definite recommendations cannot be made.

In conclusion, with the high incidence of CRLM and the increase in the number of hepatic resections, we need well-defined prognostic factors, studied in homogeneous patient populations, to optimize diagnostic work-up. This review identifies several of these factors.

Introduction

Liver metastases constitute the major cause of death in colorectal cancer patients, with an overall survival rate in untreated patients of <10 months.[1, 2] Surgery is the only way to achieve long-term survival, with 5-year survival rates ranging from 40% to 60%.[3-6] These survival rates are almost the same as those of patients with TNM stage III colorectal cancer.

Because of improvements in radiological imaging techniques, surgical techniques, and perioperative care, and the availability of effective systemic therapy, increasing numbers of patients are being selected for resection of their colorectal liver metastases (CRLMs).[1, 7, 8] However, there is no clear consensus on the resectability of liver metastases.[9] Several clinical scoring systems have been developed for patient selection and to predict overall survival (OS) after liver resection, with size and number, and the interval between the treatment of the primary tumour and the development of CRLM, as important prognostic items.[4, 10, 11] In addition to the clinical scoring systems, it seems highly probable that molecular and histopathological features of resected CRLMs could have potential value in the selection of patients who may benefit from adjuvant systemic treatment. For primary colorectal carcinoma, many histological prognostic factors have been identified, and therapeutic decisions concerning adjuvant systemic therapy are made on the basis of these histopathological findings.[12] However, in reporting metastatic lesions, there is usually only confirmation of the malignancy, and the (lack of) involvement of resection margins is mentioned. More exhaustive diagnostic reporting of the metastases might be warranted. In this article, we critically review potentially important prognostic factors for resected CRLM and focus specifically on histopathological features.

Resection margin

The surgical margin of liver metastases is an important prognostic factor. Patients with positive margins usually have a worse outcome.[3, 13-15] Although patients with a negative resection margin have an improved outcome, the significance of the width of the negative margins remains controversial. Traditionally, anatomical resection was proposed in liver surgery in order to achieve minimal margins of 10 mm.[16] Dhir *et al*.[17] conducted a meta-analysis of 18 studies with 4821 patients, to determine whether negative resection margins of ≥10 mm confer a survival advantage over negative resection margins of <10 mm. The 5-year overall survival rate for the subgroup with margins of ≥10 mm was 46% (95% CI 44–48%), as compared with 38% in the subgroup with margins of <10 mm (95% CI 36–40%), suggesting that a margin of 10 mm should be pursued. However, owing to anatomical restrictions, these margins cannot always be achieved, and they might not always be necessary, especially in the era of neoadjuvant chemotherapy.[9, 18] Ayez *et al*. described similar disease-free

and overall survival rates in patients with either R0 or R1 resection treated with neoadjuvant chemotherapy,[18] suggesting that microscopic tumour remnants after treatment are no longer of major importance, and that survival after neoadjuvant chemotherapy is more related to tumour biology than to resection margins. A molecular approach in a limited number of patients (n = 12) showed that DNA of tumour cells could be detected 4 mm from the tumour;[19] biopsies of surrounding liver tissue at 8, 12 and 16 mm from the tumour border showed no tumour DNA, suggesting that a resection margin of >4 mm is adequate.

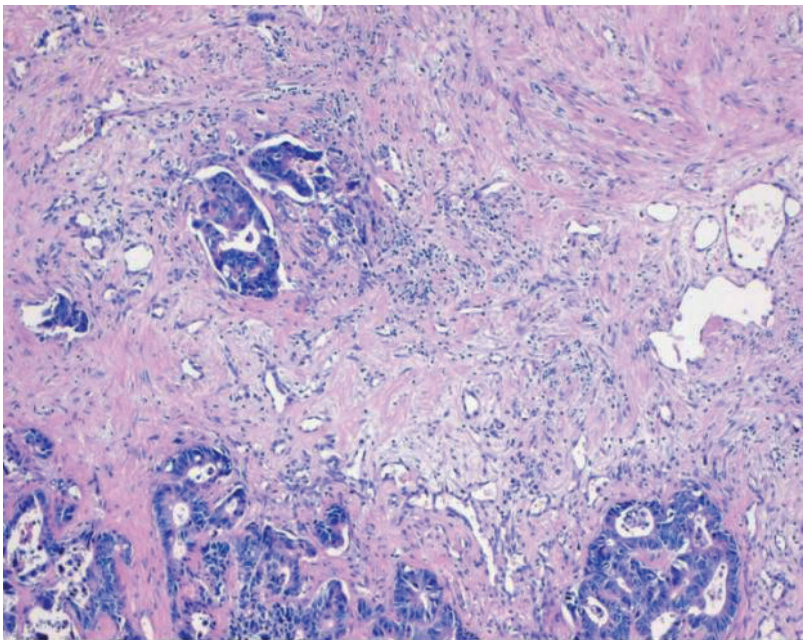
Intrahepatic invasion

Theoretically, metastatic tumour can spread within the liver by different pathways. Tumour cells might use pre-existing portal and hepatic veins, lymphatic vessels, bile ducts and nerves for dissemination within and outside the liver. Multiple studies have investigated the incidence of intrahepatic spread;[20-29] however, the exact definitions of intrahepatic spread and the methods used to detect it were not described in most articles. Only two studies defined the different forms of intrahepatic spread,[24, 28] and one of these also specified the methods used for detection.[28] The study by Sasaki *et al*. defined portal vein, hepatic vein and bile duct invasion as cancer cells growing in the lumen of a vessel or bile duct branches within the liver.[24] Intrahepatic lymphatic invasion was described as cancer cells in luminal structures in the portal area lined by endothelial cells. The study by Korita *et al*. defined lymphatic invasion as single tumour cells or cell clusters visible within vessels that show immunoreactivity for D2-40 monoclonal antibody.[28] Other forms of intrahepatic spread (portal vein, hepatic vein, and sinusoidal and bile duct invasion) were not defined in this study.[28] With standard H&E staining, lymphatic vessels cannot be distinguished from blood vessels (Figure 1A). D2-40 staining of the lymphatic vessels could be helpful in detecting tumour cells within lymphatic vessels (Figure 1B). This staining was used in one study,[28] and, because other studies did not mention the method used to visualize lymphatic invasion, it remains unclear how they differentiated between invasion of blood vessels and invasion of lymphatic vessels.

Portal vein invasion

Eight studies have investigated the incidence of portal vein invasion in CRLM (Figure 2).[20-24, 26, 28, 29] These studies included 607 patients in total. The mean incidence of portal vein invasion was 26.2% (range 10–49%). Four studies (n = 247) reported data on 5-year OS in patients with and without portal vein invasion.[20, 23, 24, 29] Although the sample sizes of these studies are relatively small, leading to significant heterogeneity, there seems to be better overall survival in patients without portal vein invasion (RR 1.77, 95% CI 1.26–2.47) (Figure 3A).

A



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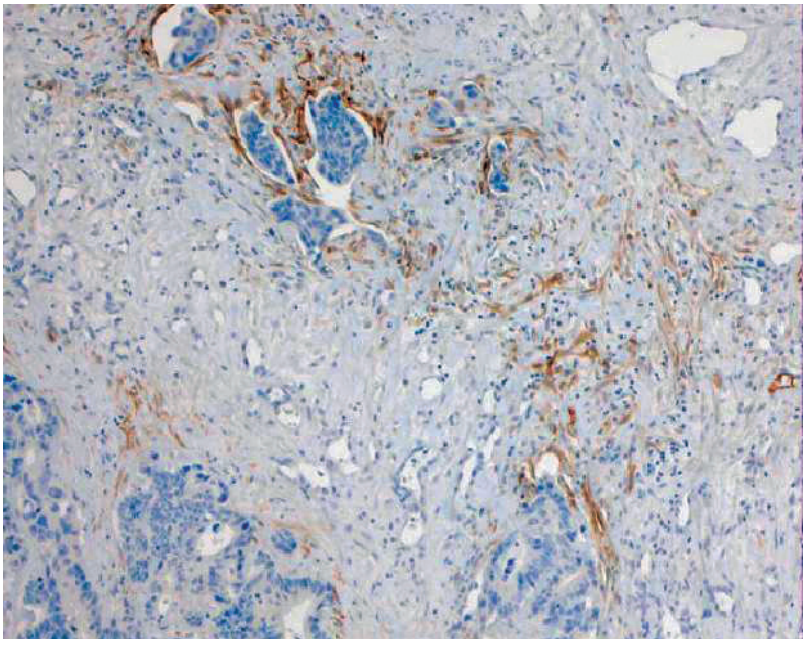


Figure 1. A, Haematoxylin and eosin staining at the border of a tumour. Small vessels are present, but differentiation between blood and lymphatic vessels is difficult. **B,** Immunohistochemical staining with the D2-40 monoclonal antibody reveals tumour cells within lymphatic vessels.

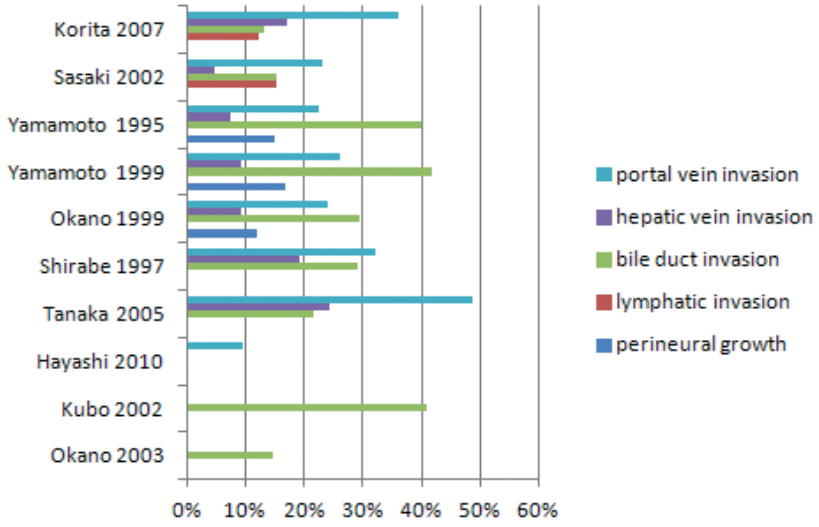


Figure 2. Frequency of different types of intrahepatic spread.

Hepatic vein invasion

Seven studies investigated the incidence of hepatic vein invasion (Figure 2).[20-24, 26, 2] They included 523 patients, 62 of whom had hepatic vein invasion (11.9%, range 5–24%). Three studies (n = 192) investigated the impact of hepatic vein invasion on 5-year OS.[20, 23, 24] Because of the small number of patients, the impact of hepatic vein invasion remains unclear (RR 1.53, 95% CI 0.81–2.89) (Figure 3B).

Lymphatic invasion

Two studies investigated the incidence of lymphatic invasion (Figure 2),[24, 28] with a total of 170 patients. Lymphatic invasion was found in 12% and 15% of CRLMs. Both studies showed a negative impact of lymphatic invasion on survival (RR 1.66, 95% CI 1.42–1.95) (Figure 3C).

Bile duct invasion

Nine studies investigated the incidence of bile duct invasion (Figure 2).[21-28] These studies included 781 patients, 30.2% of whom had bile duct invasion (range 13–42%). Five studies (n = 346) reported data on 5-year OS in patients with and without bile duct invasion of the CRLMs.[20, 23-26] There seems to be no correlation between bile duct invasion of the CRLMs and clinical outcome (1.22, 95% CI 0.94–1.58) (Figure 3D).

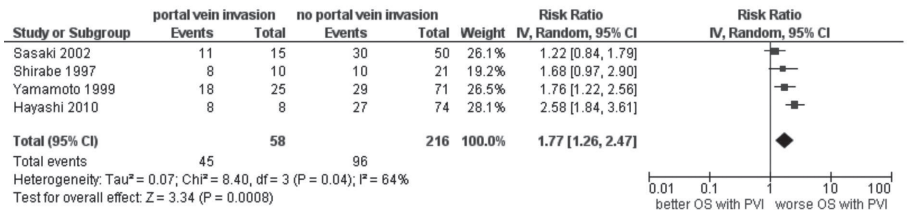


Figure 3A

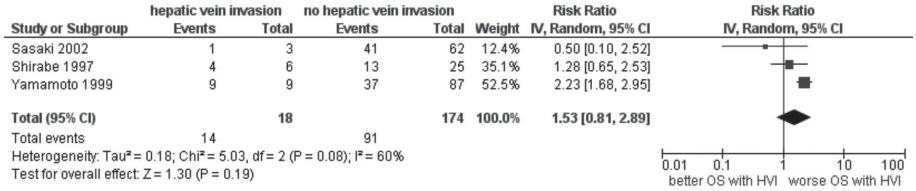


Figure 3B

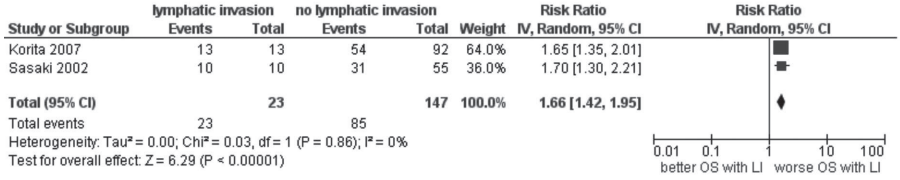


Figure 3C

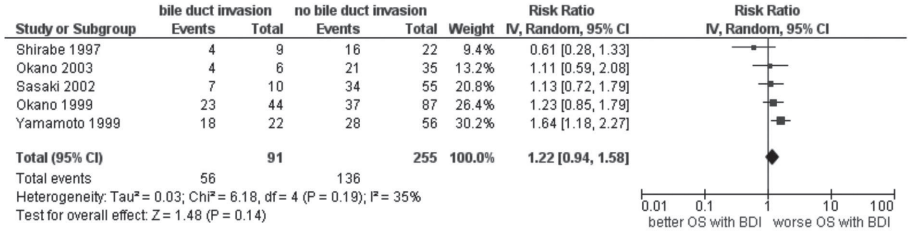


Figure 3D

Figure 3. Forest plots for the prognostic value of intrahepatic spread. **A,** Portal vein invasion (PVI). **B,** Hepatic vein invasion (HVI). **C,** Lymphatic invasion (LI). **D,** Bile duct invasion (BDI). CI, confidence interval; OS, overall survival.

Perineural invasion

Three studies investigated the incidence of perineural growth in CRLM (Figure 2).[20, 21, 26] Perineural invasion was found in 40 of 285 patients (14.0%) (range 12–17%). One study, by Yamamato,[20] investigated the impact of perineural invasion on 5-year OS, and found that it was negative.

Presence of micrometastases

In analogy with primary colorectal tumours, micrometastases may occur in liver metastases. Micrometastases are defined as discrete microscopic cancerous lesions ranging from a single cell to clusters of cells within the liver parenchyma or portal tracts surrounding the dominant macroscopic hepatic tumour. Yokoyama *et al*. detected micrometastases in 32 of 46 patients, by using CK20 staining.[30] Patients with micrometastases were reported to have a higher probability of intrahepatic recurrence and poorer survival. They had a 10-year survival rate of 21.9%, versus 64.3% for patients without micrometastases. In the definition used by Yokoyama, there is an overlap between micrometastases and intrahepatic spread.

Presence of a fibrous capsule

The presence of a fibrous capsule has been recognized as a favourable prognostic factor in hepatocellular carcinomas.[31] A study by Okano *et al*. investigated the prognostic value of a fibrous capsule in liver metastases of colorectal origin.32 Fibrotic tissue between the tumour and surrounding hepatic parenchyma was classified as thick (≥10 layers of collagen bundles) or thin (several layers of collagen bundles). Fibrotic tissue was observed in 61% of patients, and was associated with improved survival. Patients with a thick pseudocapsule had 5-year survival rates of 88%, as compared with 64% in patients with a thin pseudocapsule and 31% in patients without a pseudocapsule. Yamamoto *et al*. confirmed the prognostic value of a fibrous pseudocapsule after hepatectomy for colorectal metastases.[20] A thick pseudocapsule was associated with a 5-year survival rate of 71%, a thin pseudocapsule with a 5-year survival rate of 63%, and the absence of a pseudocapsule with a 5-year survival rate of only 19%.

Response to neoadjuvant chemotherapy

Tumour regression grading

Five studies investigated the histological response of liver metastases to preoperative chemotherapy (Table 1).[33-37] All studies showed some effect on survival, but different grading systems were employed to assess pathological response to chemotherapy. Two studies used complete pathological response versus all other responses, including non-responses.[33, 37] In the study by Adam *et al*.,[33] each nodule was sampled for histological examination, one block for each centimetre of diameter of the nodule.

Complete pathological response was defined as the absence of any viable tumour cells irrespective of the proportions of necrosis and fibrosis. In the study by Tanaka *et al*.,[37] complete pathological response was defined as the absence of any viable tumour cells, irrespective of the proportions of necrosis and fibrosis, in the largest cut surface of macroscopically confirmed metastatic tumours, or at sites in resected specimens corresponding to areas where metastases were initially detected in preoperative images. A limitation of those grading systems is the inability to identify partial responders who may also have better survival. In addition, even complete pathological response is sensitive to bias, because it depends on the number of lesions assessed and the interpretation of the pathologist. Other pathological response grading systems are based on a semi-quantitative analysis of the proportion of viable cancer cells remaining, and are therefore subject to variability in interpretation.[34, 35] It is impossible to determine the percentage of remaining cancer cells, because there are no data on the baseline percentage of tumour cells prior to chemotherapy. Moreover, liver metastases frequently show necrosis surrounded by adenocarcinoma cells, regardless of neoadjuvant therapy, and the value of necrosis has not been established. A large area of necrosis will decrease the percentage of remaining cancer cells in most grading systems, and does not represent the efficacy of chemotherapy.

The grading system of Rubbia-Brandt *et al*. seems to be the most accurate, because it takes into account the necrotic areas, fibrotic areas, and residual cancer cells.[36] Moreover, for its establishment, chemotherapy-naïve tumours were used as a control. Although this seems essential, this is the only study to incorporate such controls. The system is a modified version of the tumour regression scheme of Mandard *et al*.[38] for oesophageal carcinomas. The score identifies five tumour regression grades (TRGs) on the basis of the presence of residual tumour cells and the extent of fibrosis. TRG1 corresponds to the absence of tumour cells replaced by abundant fibrosis; TRG2 to rare residual tumour cells scattered throughout abundant fibrosis; TRG3 to a greater number of residual tumour cells with predominant fibrosis; TRG4 to a large number of tumour cells predominating over fibrosis; and TRG5 to tumour cells without fibrosis.

Tumour thickness at the tumour–normal interface

Maru *et al*. measured the tumour thickness at the tumour–normal interface of 103 patients with CRLMs resected after preoperative chemotherapy.[39] The recurrence-free survival rates were 70% for patients with a tumour thickness of <0.5 mm, 51% for patients with a tumour thickness between 0.5 mm and 5 mm, and 35% for patients with a tumour thickness of ≥5 mm. A limitation of this study is that the role of tumour thickness in chemotherapy-naïve liver metastases was not investigated. Therefore, it could be that tumour thickness at the tumour–normal interface is a prognostic factor, rather than a predictive factor for response to chemotherapy.

Number of lesions to be assessed for chemotherapy response

There is conflicting literature on the histological response to chemotherapy of different liver metastases within one patient. Rubbia-Brandt *et al*. showed 89% concordance in histological response.[36] However, Tanaka *et al*. found that, within the same patient, some liver metastases showed a complete response, whereas other metastases did not.[37] Better survival was demonstrated in patients with a pathologically complete response in at least one liver metastasis than in patients with no pathologically complete responses. The best overall survival rate was reached in patients with all lesions showing complete responses. Until there are more data on the variation in histological response of multiple liver metastases within a patient, histological sampling of each lesion is recommended to assess the pathological response to chemotherapy.

Table 1. Correlation of histological response of colorectal liver metastases after neoadjuvant chemotherapy with 5-year overall survival

author	year	no of pts	neo-adjuvant chemo-therapy	surgery alone	grading system histological response to chemotherapy	5y OS	p-value
Adam	2008	767	767	-	complete pathological response (n=29)	76%	
					no complete pathological response (n=738)	45%	0.004
Blazer	2008	271	271	-	complete response (no residual cancer cells) (n=25)	75%	0.037
					major response (1%-49% residual cancer cells) (n=97)	56%	
					minor response (>50% residual cancer cells) (n=149)	33%	0.028
Chan	2010	50	50	-	strong pathological response (<10% viable tumor cells) (n=17)	80%	0.019
					weak pathological response (>10% viable tumor cells) (n=33)	51%	
Rubbia-Brandt	2007	181	112	69	major or complete histological tumor regression (TRG 1+2) (n=27)	41%	
					partial histological tumor regression (TRG 3) (n=36)	38%	0.0003
					no histological tumor regression (TRG 4+5) (n=49)	9%	0.0019
Tanaka	2009	63	63	-	complete pathological response (n=23)	69%	
					no complete pathological response (n=40)	8%	0.001

Discussion

The benefits of hepatic resection for survival in patients with CRLMs are well established; however, there is still a challenge in selecting the right patients and preventing recurrence. Macroscopic features of resected metastases, such as size, number, and synchronous or metachronous disease, are important prognostic factors in many studies. These, together with staging of the primary tumour, are factors in clinical risk scores (CRSs), such as that of Fong *et al*.[4] This CRS is widely used to stratify patients into high-risk and low-risk groups for overall survival.[40, 41]

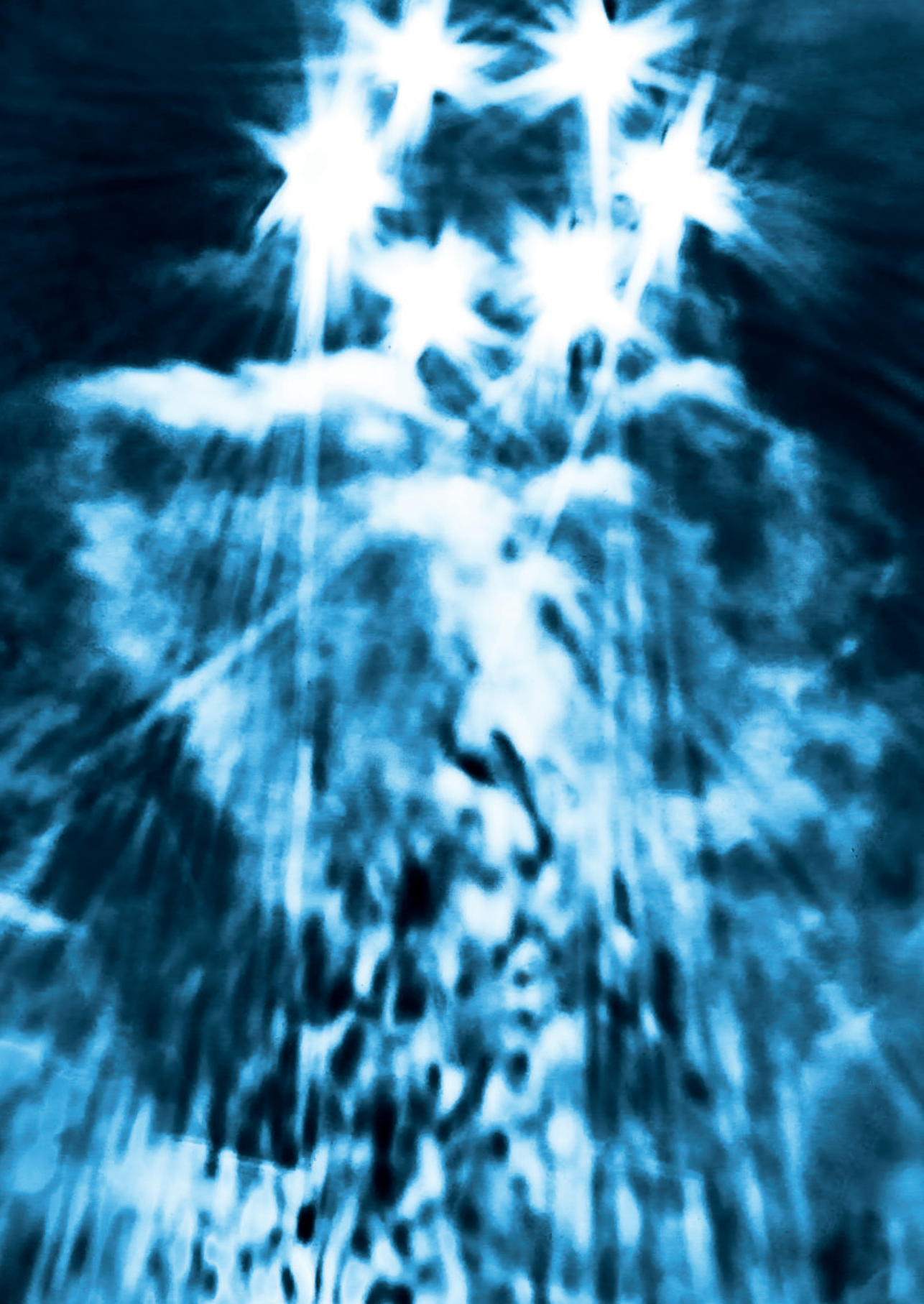
In analogy with primary tumours, histopathological factors, such as vascular or perineural invasion and response to chemotherapy, have been investigated in CRLMs. Whereas studies in primary colorectal cancer typically consist of large series of patients, in which well-defined histological factors are investigated, pathological research of liver metastases is still in its infancy. Potentially useful factors have been investigated in relatively small, sometimes heterogeneous, groups of patients, but the evaluation of promising features, such as intrahepatic invasion and tumour regression grade, will require study of larger series with, for investigation of tumour response to neoadjuvant chemotherapy, use of well-defined grading systems with chemotherapy-naive liver metastases as controls.

With the high frequency of CRLMs and the increasing number of hepatic resections, there is a need for well-defined prognostic histopathological factors. Prospective studies of populations of patients with CRLMs are warranted to evaluate prognostic and/or predictive factors, such as histopathological features and (novel) biomarkers, in order to assist treatment decisions.

References

1. Dexiang Z, Li R, Ye W *et al.* Outcome of patients with colorectal liver metastasis: Analysis of 1,613 consecutive cases. *Ann Surg Oncol* 2012.
2. Morris EJ, Forman D, Thomas JD *et al.* Surgical management and outcomes of colorectal cancer liver metastases. *Br J Surg* 2010;**97**;1110-1118.
3. Are C, Gonen M, Zazzali K *et al.* The impact of margins on outcome after hepatic resection for colorectal metastasis. *Ann Surg* 2007;**246**;295-300.
4. Fong Y, Fortner J, Sun RL, Brennan MF, Blumgart LH. Clinical score for predicting recurrence after hepatic resection for metastatic colorectal cancer: Analysis of 1001 consecutive cases. *Ann Surg* 1999;**230**;309-318; discussion 318-321.
5. Muratore A, Ribero D, Zimmitti G, Mellano A, Langella S, Capussotti L. Resection margin and recurrence-free survival after liver resection of colorectal metastases. *Ann Surg Oncol* 2010;**17**;1324-1329.
6. Pawlik TM, Scoggins CR, Zorzi D *et al.* Effect of surgical margin status on survival and site of recurrence after hepatic resection for colorectal metastases. *Ann Surg* 2005;**241**;715-722, discussion 722-714.
7. de Haas RJ, Wicherts DA, Andreani P *et al.* Impact of expanding criteria for resectability of colorectal metastases on short- and long-term outcomes after hepatic resection. *Ann Surg* 2011;**253**;1069-1079.
8. Nordlinger B, Sorbye H, Glimelius B *et al.* Perioperative chemotherapy with folfox4 and surgery versus surgery alone for resectable liver metastases from colorectal cancer (eortc intergroup trial 40983): A randomised controlled trial. *Lancet* 2008;**371**;1007-1016.
9. de Haas RJ, Wicherts DA, Flores E, Azoulay D, Castaing D, Adam R. R1 resection by necessity for colorectal liver metastases: Is it still a contraindication to surgery? *Ann Surg* 2008;**248**;626-637.
10. Iwatsuki S, Dvorchik I, Madariaga JR *et al.* Hepatic resection for metastatic colorectal adenocarcinoma: A proposal of a prognostic scoring system. *J Am Coll Surg* 1999;**189**;291-299.
11. Nordlinger B, Guiguet M, Vaillant JC *et al.* Surgical resection of colorectal carcinoma metastases to the liver. A prognostic scoring system to improve case selection, based on 1568 patients. Association francaise de chirurgie. *Cancer* 1996;**77**;1254-1262.
12. Zaniboni A, Labianca R, Gruppo Italiano per lo Studio e la Cura dei Tumori del D. Adjuvant therapy for stage ii colon cancer: An elephant in the living room? *Ann Oncol* 2004;**15**;1310-1318.
13. Pawlik TM, Vauthey JN. Surgical margins during hepatic surgery for colorectal liver metastases: Complete resection not millimeters defines outcome. *Ann Surg Oncol* 2008;**15**;677-679.
14. Hamady ZZ, Cameron IC, Wyatt J, Prasad RK, Toogood GJ, Lodge JP. Resection margin in patients undergoing hepatectomy for colorectal liver metastasis: A critical appraisal of the 1cm rule. *Eur J Surg Oncol* 2006;**32**;557-563.
15. Nuzzo G, Giuliante F, Ardito F *et al.* Influence of surgical margin on type of recurrence after liver resection for colorectal metastases: A single-center experience. *Surgery* 2008;**143**;384-393.
16. Scheele J, Stang R, Altendorf-Hofmann A, Paul M. Resection of colorectal liver metastases. *World J Surg* 1995;**19**;59-71.
17. Dhir M, Lyden ER, Wang A, Smith LM, Ullrich F, Are C. Influence of margins on overall survival after hepatic resection for colorectal metastasis: A meta-analysis. *Ann Surg* 2011;**254**;234-242.
18. Ayez N, Lalmahomed ZS, Eggermont AM *et al.* Outcome of microscopic incomplete resection (r1) of colorectal liver metastases in the era of neoadjuvant chemotherapy. *Ann Surg Oncol* 2012;**19**;1618-1627.
19. Holdhoff M, Schmidt K, Diehl F *et al.* Detection of tumor DNA at the margins of colorectal cancer liver metastasis. *Clin Cancer Res* 2011;**17**;3551-3557.
20. Yamamoto J, Shimada K, Kosuge T, Yamasaki S, Sakamoto M, Fukuda H. Factors influencing survival of patients undergoing hepatectomy for colorectal metastases. *Br J Surg* 1999;**86**;332-337.
21. Yamamoto J, Sugihara K, Kosuge T *et al.* Pathologic support for limited hepatectomy in the treatment of liver metastases from colorectal cancer. *Ann Surg* 1995;**221**;74-78.

22. Tanaka K, Shimada H, Kubota K *et al.* Effectiveness of prehepatectomy intra-arterial chemotherapy for multiple bilobar colorectal cancer metastases to the liver: A clinicopathologic study of peritumoral vasculobiliary invasion. *Surgery* 2005;**137**;156-164.
23. Shirabe K, Takenaka K, Gion T *et al.* Analysis of prognostic risk factors in hepatic resection for metastatic colorectal carcinoma with special reference to the surgical margin. *Br J Surg* 1997;**84**;1077-1080.
24. Sasaki A, Aramaki M, Kawano K, Yasuda K, Inomata M, Kitano S. Prognostic significance of intrahepatic lymphatic invasion in patients with hepatic resection due to metastases from colorectal carcinoma. *Cancer* 2002;**95**;105-111.
25. Okano K, Maeba T, Moroguchi A *et al.* Lymphocytic infiltration surrounding liver metastases from colorectal cancer. *J Surg Oncol* 2003;**82**;28-33.
26. Okano K, Yamamoto J, Moriya Y *et al.* Macroscopic intrabiliary growth of liver metastases from colorectal cancer. *Surgery* 1999;**126**;829-834.
27. Kubo M, Sakamoto M, Fukushima N *et al.* Less aggressive features of colorectal cancer with liver metastases showing macroscopic intrabiliary extension. *Pathol Int* 2002;**52**;514-518.
28. Korita PV, Wakai T, Shirai Y *et al.* Intrahepatic lymphatic invasion independently predicts poor survival and recurrences after hepatectomy in patients with colorectal carcinoma liver metastases. *Ann Surg Oncol* 2007;**14**;3472-3480.
29. Hayashi M, Inoue Y, Komeda K *et al.* Clinicopathological analysis of recurrence patterns and prognostic factors for survival after hepatectomy for colorectal liver metastasis. *BMC Surg* 2010;**10**;27.
30. Yokoyama N, Shirai Y, Ajioka Y, Nagakura S, Suda T, Hatakeyama K. Immunohistochemically detected hepatic micrometastases predict a high risk of intrahepatic recurrence after resection of colorectal carcinoma liver metastases. *Cancer* 2002;**94**;1642-1647.
31. Ng IO, Lai EC, Ng MM, Fan ST. Tumor encapsulation in hepatocellular carcinoma. A pathologic study of 189 cases. *Cancer* 1992;**70**;45-49.
32. Okano K, Yamamoto J, Kosuge T *et al.* Fibrous pseudocapsule of metastatic liver tumors from colorectal carcinoma. Clinicopathologic study of 152 first resection cases. *Cancer* 2000;**89**;267-275.
33. Adam R, Wicherts DA, de Haas RJ *et al.* Complete pathologic response after preoperative chemotherapy for colorectal liver metastases: Myth or reality? *J Clin Oncol* 2008;**26**;1635-1641.
34. Blazer DG, 3rd, Kishi Y, Maru DM *et al.* Pathologic response to preoperative chemotherapy: A new outcome end point after resection of hepatic colorectal metastases. *J Clin Oncol* 2008;**26**;5344-5351.
35. Chan G, Hassanain M, Chaudhury P *et al.* Pathological response grade of colorectal liver metastases treated with neoadjuvant chemotherapy. *HPB (Oxford)* 2010;**12**;277-284.
36. Rubbia-Brandt L, Giostra E, Brezault C *et al.* Importance of histological tumor response assessment in predicting the outcome in patients with colorectal liver metastases treated with neo-adjuvant chemotherapy followed by liver surgery. *Ann Oncol* 2007;**18**;299-304.
37. Tanaka K, Takakura H, Takeda K, Matsuo K, Nagano Y, Endo I. Importance of complete pathologic response to prehepatectomy chemotherapy in treating colorectal cancer metastases. *Ann Surg* 2009;**250**;935-942.
38. Mandard AM, Dalibard F, Mandard JC *et al.* Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. Clinicopathologic correlations. *Cancer* 1994;**73**;2680-2686.
39. Maru DM, Kopetz S, Boonsirikamchai P *et al.* Tumor thickness at the tumor-normal interface: A novel pathologic indicator of chemotherapy response in hepatic colorectal metastases. *Am J Surg Pathol* 2010;**34**;1287-1294.
40. Mala T, Bohler G, Mathisen O, Bergan A, Soreide O. Hepatic resection for colorectal metastases: Can preoperative scoring predict patient outcome? *World J Surg* 2002;**26**;1348-1353.
41. Reissfelder C, Rahbari NN, Koch M *et al.* Validation of prognostic scoring systems for patients undergoing resection of colorectal cancer liver metastases. *Ann Surg Oncol* 2009;**16**;3279-3288.



Chapter 2

Lymphatic invasion is an independent adverse prognostic factor in patients with colorectal liver metastasis

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Abstract

For a selection of patients with colorectal liver metastases (CRLM), liver resection is a curative option. In order to predict long-term survival, clinicopathologic risk scores have been developed, but little is known about histologic factors and their prognostic value for disease-free and overall survival. The objective of the present study was to assess possible prognostic histologic factors in patients with solitary CRLM treated with liver resection who did not receive neoadjuvant treatment.

Patients with solitary CRLM who underwent liver resection between 1992 and 2011 were evaluated for clinical prognostic factors. Histologic analyses on tumor thickness at the tumor-normal interface, presence of a fibrotic capsule, intrahepatic vascular invasion, lymphatic invasion, or bile duct invasion and perineural growth were performed, using immunohistochemistry.

A total of 124 patients were analyzed with a median follow-up of 41 months (range 1–232 months). There was no association between histologic factors and disease-free survival in multivariate analysis. In multivariate analysis, intrahepatic lymphatic invasion was associated with a decreased overall survival (41.9 vs. 61.0 months; $p = 0.041$), especially in combination with vascular invasion ($n = 15$) (28.1 vs. 62.2 months; $p < 0.0001$). In addition, size over 50 mm (29.2 vs. 65.9 months; $p = 0.004$) and interval less than 12 months between resection of the primary tumor and diagnosis of liver metastasis (49.0 vs. 91.5 months: $p = 0.019$) were also independent adverse prognostic factors.

Intrahepatic lymphatic invasion, especially in combination with vascular invasion, is an important adverse prognostic factor for overall survival in patients with solitary CRLM after liver resection.

Introduction

Colorectal cancer is one of the leading causes of cancer death worldwide as a result of its considerable risk of development of metastases.[1] When metastatic disease is confined to the liver, partial liver resection is the only curative therapeutic option, with 5-year overall survival (OS) percentages between 20 and 60%, depending on patient and tumor characteristics.[2-4] In order to explain these varying survival rates, different clinicopathologic risk scores have been developed. In many of these risk scores, nodal status of the primary tumor, size and number of the colorectal liver metastases (CRLM), disease-free interval from treatment of the primary until detection of the CRLM, and preoperative level of carcinoembryonic antigen (CEA) are combined to predict long-term survival.[5-9] These scoring systems are relevant with respect to prediction of survival, but to our knowledge, they have not been used for risk stratification in controversial areas such as the administration of neoadjuvant or adjuvant systemic therapy or surveillance.

In primary colorectal cancer histologic factors such as extramural venous invasion, perineural growth, lymphatic invasion, angioinvasion, and diffuse growth pattern have been associated with poorer survival outcomes.[10, 11] Extramural venous invasion in particular is considered a poor prognostic factor, and as a result, patients with extramural venous invasion in stage II colon cancer are considered candidates for adjuvant systemic treatment.[12] Very little is known about the impact of histologic features of colorectal liver metastases on OS, as described in a recent review.[13] Vascular invasion, bile duct invasion, or lymphatic invasion by tumor cells in CRLM have all been suggested as prognostic factors for long-term survival.[5, 14-23] Perineural growth, the presence of a fibrous capsule, and tumor thickness at the tumor–normal interface have also been linked to survival in patients with CRLM.[14, 15, 19, 24-26] Variations in definitions and selection of patients have limited the impact of these studies. Furthermore, none of these previous studies has evaluated multiple histologic factors of the liver resection specimens, in combination with established risk scores in a homogenous group of patients. Most studies included patients who underwent neoadjuvant therapy as well as chemotherapy-naive patients, patients with multiple liver metastases, or patients with extrahepatic disease.[5, 14-21, 23, 24] The results of these previous studies might be biased because of the known changes in histologic features observed in liver metastases after systemic therapy, and the possible heterogeneous nature of multiple metastases.[27-30]

The objective of the current study was to assess possible prognostic histologic factors for long-term survival in patients with solitary colorectal liver metastases who underwent a complete (R0) liver without neoadjuvant systemic therapy.

Materials and methods

Patients

Patients were identified who underwent complete (R0) liver resection for a solitary CRLM between 1992 and 2011 in a tertiary referral hospital. R0 resections were defined as liver resections with clear resection margins in patients who did not have evidence of disease in any other locations.

Demographics and clinicopathologic factors with regard to the primary tumor, as well as the liver metastasis, were collected per patient. Special attention was given to the four different items from the clinical risk score according to Fong et al.: nodal status of the primary tumor; preoperative CEA level and size of the metastasis, and interval between resection of the primary tumor and diagnosis of CRLM.[9] It is unknown whether systemic treatment influences the presence of certain histopathology factors and therefore patients who were treated with neoadjuvant systemic therapy were excluded from the current study. Patients who died from postoperative complications, defined as within 30 days after liver resection, were also excluded. Patients underwent follow-up according to our current Dutch follow-up guidelines, with regular outpatient visits, CEA testing and computed tomographic scans of chest and abdomen.

Histopathology

R0 liver resection specimens with a solitary CRLM were selected from the archive. Routine workup consisted of sampling of macroscopically normal liver tissue, invasive front of the metastasis, and additional tumor blocks, depending on the size of metastasis. Slide revision was performed independently by two investigators (JdR, NK). Discrepancies were resolved by simultaneous re-examination of the slides by both investigators using a two-headed microscope. In case of discrepancy, the senior pathologist (IN) made the final call.

Tumor thickness at the tumor–normal interface was determined in routine slides. Tumor–normal interface was defined as the interface between tumor and normal liver tissue, as described by Maru et al. and validated by others.[26, 31, 32] In all tumors, tumor thickness was measured with a ruler at multiple foci, and maximum tumor thickness was used and defined as uninterrupted layers of tumor cells without admixed fibrotic stroma, acellular mucin, or nonneoplastic liver parenchyma. The median tumor thickness at tumor–normal interface was used to divide the patient group in a group with a larger and a smaller layer of vital tumor cells (Figure 1).

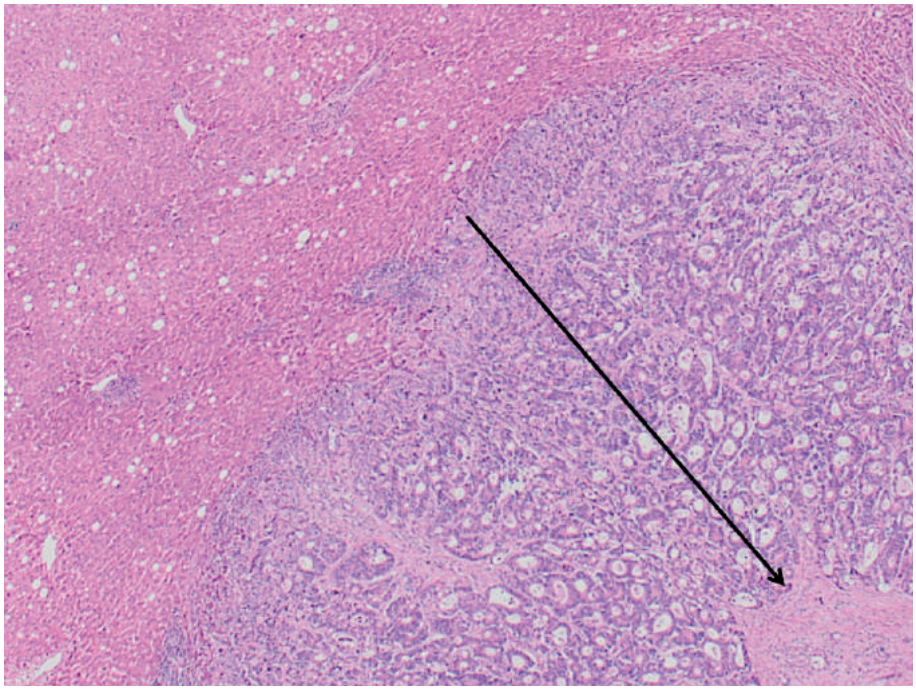


Figure 1. Tumor thickness at tumor–normal interface; arrow indicates correct measurement with uninterrupted layer of tumor cells. Original magnification, x10

The presence of a fibrotic capsule around the metastasis was evaluated in routine slides. The fibrous tissue between tumors and liver parenchyma was classified as absent (no fibrous tissue observed) or present: tumor was separated from the liver parenchyma by several layers of collagen bundles in histologic sections (Figure 2).

Immunohistochemistry and scoring methods

Immunohistochemistry was performed as previously described.[33] Perineural growth was defined as a nerve, identified by S-100 staining, being surrounded by tumor cells for at least three quarters of the circumference and was scored as being present or absent (Figure 3A). Lymphatic invasion was defined as single tumor cells or cell clusters visible within vessels that showed immunoreactivity for D2-40 but not for CD31. Lymphatic invasion was scored as being present or absent (Figure 3B).Vascular invasion was defined as single tumor cells or cell clusters visible within vessels that showed immunoreactivity for CD31 but not for D2-40. It was scored as being present or absent (Figure 3C). Bile duct invasion was defined as single tumor cells or cell clusters (CK7 negative) visible within bile ducts that showed immunoreactivity for CK7. It was also scored as being present or absent (Figure 3D).

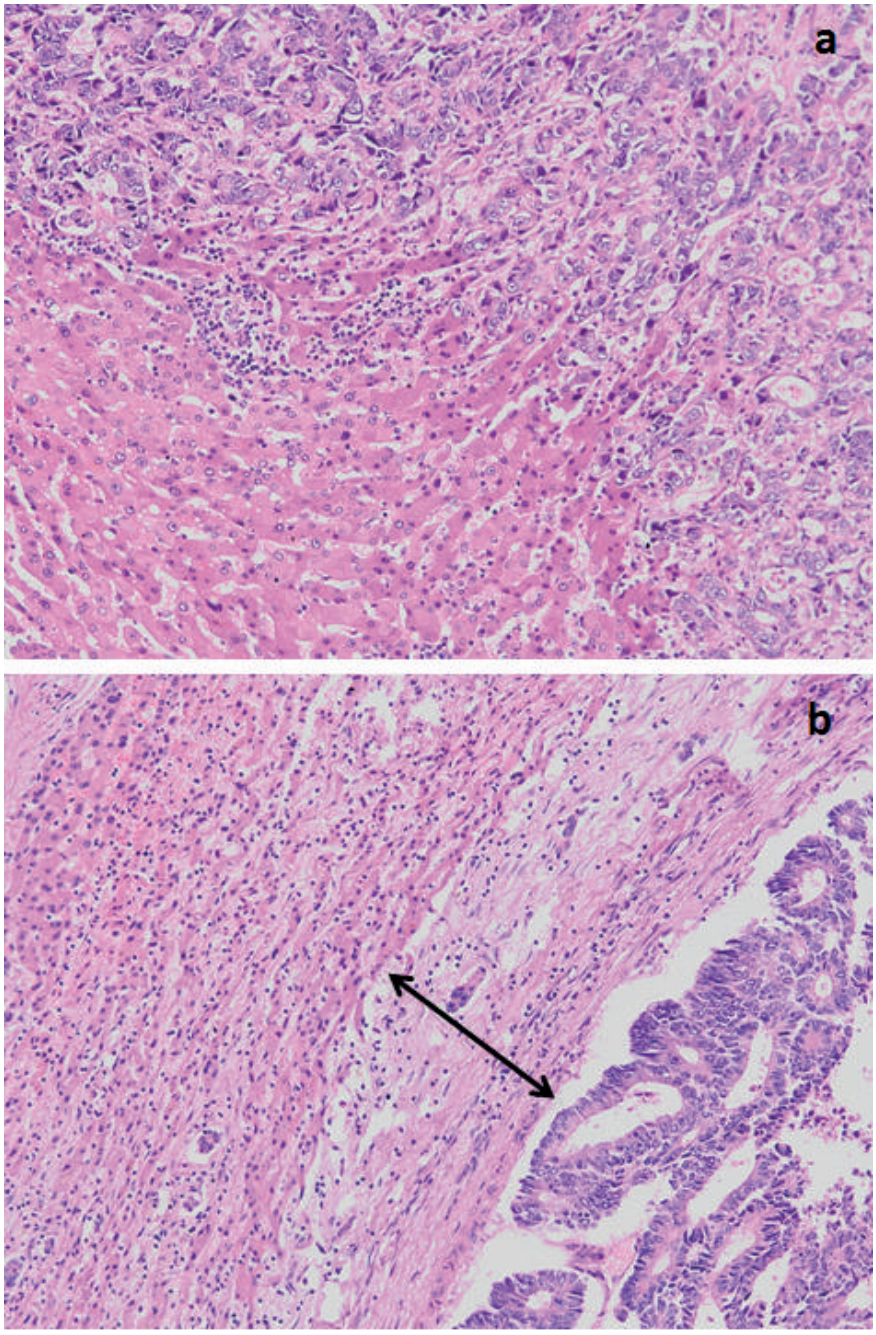


Figure 2. a Colorectal liver metastasis without fibrous capsule. Original magnification, x20. b Colorectal liver metastasis with fibrous capsule (arrow). Original magnification, x20

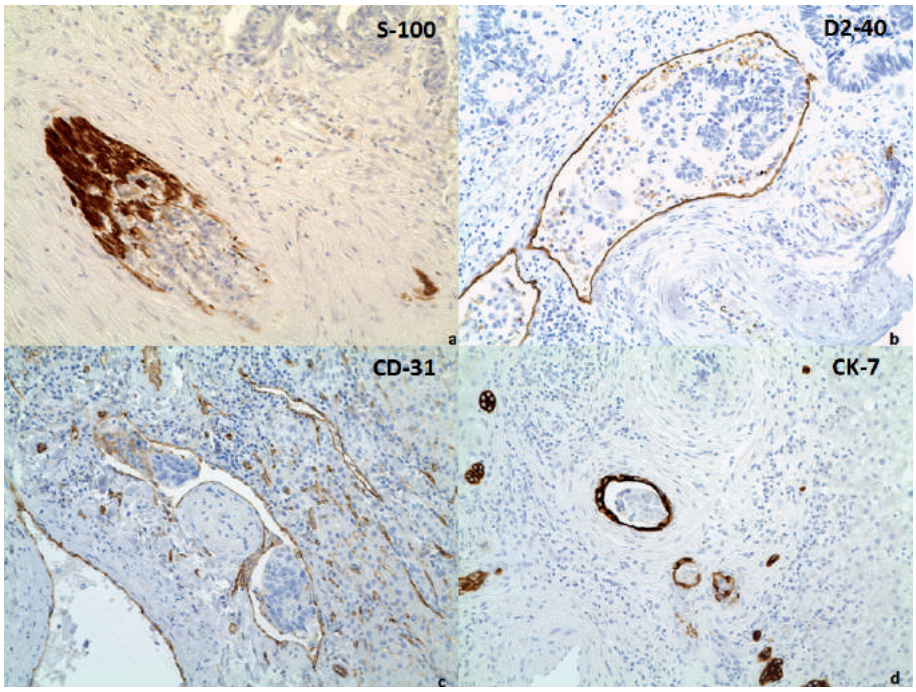


Figure 3. Different forms of intrahepatic invasion by tumor cells.
a Perineural growth showing S-100 reactivity.
b Lymphatic invasion showing D2-40 reactivity.
c Vascular invasion showing CD-31 reactivity.
d Bile duct invasion showing CK-7 reactivity. Original magnification, x20

Outcome

Primary outcomes were disease-free survival (DFS) and OS. DFS was defined as the interval in months between liver resection and disease recurrence, death, or last follow-up. OS was defined as the interval in months between liver resection and death or date of last followup.

Statistical analysis

Pearson's Chi square test was used to calculate correlations between the various histologic features. Survival curves were estimated by the Kaplan–Meier method and compared by log rank testing. Multivariate analysis was performed using Cox proportional hazard model, and variables were included that were associated with survival in univariate analysis with a p value of <0.10. SPSS statistical software, version 18.0 (IBM, Armonk, NY, USA) was used for all statistical analysis. A p value of <0.05 was considered statistically significant.

Results

Patients

Between January 1992 and March 2011, a total of 383 patients underwent liver resection for metastatic disease. After excluding patients with multiple metastases, 135 patients remained who were surgically treated (R0) for solitary CRLM. Eleven patients were excluded because they received neoadjuvant chemotherapy (n = 5), were lost to follow-up (n = 2), or died within 30 days after liver resection (n = 4). A total of 124 patients were eligible to be included in the current study, 76 men (61.3%) and 48 women (38.7%). Median age at time of resection was 64 years (range 40–80 years). Liver metastasis were detected at a median of 8.8 months (range 0–82 months) after resection of the primary tumor. Median size of the metastasis was 35 mm (range 10–130 mm). Median follow-up was 41 months (range 1–232 months). In the complete study population, median DFS was 28 months (range 1–228 months) with a median OS of 57 months (range 1–232 months) and a 5-year survival of 48.1%.

Histopathologic tumor features

Fibrous capsule and tumor thickness

In 34.4% of patients (n = 43), the liver metastasis was surrounded by a fibrous capsule. Presence of a fibrous capsule was not associated with DFS, but it was associated with an improved OS of 109.3 months, versus 56.7 months in patients without a fibrous capsule (p = 0.037). In multivariate analysis, presence of a fibrous capsule was not an independent risk factor for OS (Tables 1, 2). Tumor thickness at tumor–normal interface varied between 0.1 and 7.2 mm, with a median of 3 mm, and was not correlated with the size of the liver metastases (p = 0.213). Although there was a significant association of increased thickness with decreased outcome (both DFS and OS) in univariate analysis, it was no longer significant in multivariate analysis (Tables 1, 2).

Intrahepatic spread

Frequency of different forms of intrahepatic invasion varied; perineural growth (n = 11; 8.9%) and bile duct invasion (n = 11; 8.8%) were both relatively uncommon, whereas vascular and lymphatic invasion were seen more frequently (n = 46; 37.1%, respectively n = 33; 26.6%). In univariate analysis, presence of bile duct invasion was associated with improved OS (76.7 vs. 55.9 months; p = 0.048), but this was not the case in multivariate analysis (p = 0.094). Presence of intrahepatic lymphatic invasion was correlated with a decreased median OS (41.9 vs. 62.2 months, p = 0.013), which remained significant in multivariate analysis (p = 0.041).

Table 1. Relation of clinical and histologic factors with DFS after liver resection in patients with solitary CRLM

	n	%	Median DFS	UV *p* value	MV *p* value
Size (mm)					
≤50	93	75	50.1	0.002*	0.020*
>50	31	25	14.5		
CEA (ng/ml)					
≤200	121	97.6	27.5	0.508	–
>200	3	2.4	40.6		
DFI (months)					
≤12	72	58.1	27.8	0.232	–
>12	52	41.9	25.4		
Nodal state primary					
N0	54	43.5	35.7	0.446	
N+	70	56.5	27.5		–
Adjuvant therapy					
No	106	85.5	20.2	0.013*	0.025*
Yes	18	14.5	>50		
Tumor thickness at TNI (mm)					
≤3	60	48.4	>51	0.023*	0.118
>3	64	51.6	19.4		
Fibrous capsule					
Present	43	34.4	27.8	0.468	–
Absent	81	65.6	25.8		
Perineural growth					
Present	11	8.9	50.2	0.539	–
Absent	113	91.1	27.5		
Vascular invasion					
Present	46	37.1	18.0	0.055	0.287
Absent	78	62.9	40.8		
Lymphatic invasion					
Present	33	26.6	19.4	0.280	–
Absent	91	73.4	29.2		
Bile duct invasion					
Present	11	8.8	27.8	0.624	–
Absent	113	91.2	27.5		

DFS disease-free survival, *CRLM* colorectal liver metastases, *UV* univariate, *MV* multivariate, *CEA* carcinoembryonic antigen, *DFI* disease-free interval between treatment of primary tumor and detection of the CRLM, *TNI* tumor–normal interface

* $p < 0.05$ was considered statistically significant

Table 2. Relation of clinical and histologic factors with OS after liver resection in patients with solitary CRLM

	n	%	Median OS	UV *p* value	MV *p* value
Size (mm)					
≤50	93	75	65.9	0.050*	0.004*
>50	31	25	29.2		
CEA (ng/ml)					
≤200	121	97.6	57.3	0.912	–
>200	3	2.4	28.9		
DFI					
≤12	72	58.1	49.0	0.059	0.019*
>12	52	41.9	91.5		
Nodal state primary					
N0	54	43.5	61.0	0.231	–
N+	70	56.5	44.6		
Adjuvant therapy					
No	106	85.5	57.2	0.955	–
Yes	18	14.5	29.2		
Tumor thickness at TNI (mm)					
≤3	60	48.4	95.3	0.043*	0.068
>3	64	51.6	48.8		
Fibrous capsule					
Present	43	34.4	109.3	0.037*	0.240
Absent	81	65.6	56.7		
Perineural growth					
Present	11	8.9	109.3	0.652	–
Absent	113	91.1	55.9		
Vascular invasion					
Present	46	37.1	48.8	0.483	–
Absent	78	62.9	58.2		
Lymphatic invasion					
Present	33	26.6	41.9	0.013*	0.041*
Absent	91	73.4	62.2		
Bile duct invasion					
Present	11	8.8	76.7	0.048*	0.094
Absent	113	91.2	55.9		

OS overall survival, *CRLM* colorectal liver metastases, *UV* univariate, *MV* multivariate, *CEA* carcinoembryonic antigen, *DFI* disease-free interval between treatment of primary tumor and detection of the *CRLM, TNI* tumor–normal interface

* $p < 0.05$ was considered statistically significant

In the current study, no correlation between different forms of intrahepatic spread or between any of the histologic features and the various items of the clinical risk score was observed. However, there was a correlation between presence of a fibrous capsule and absence of intrahepatic vascular invasion ($p = 0.014$) and between presence of a fibrous capsule and presence of intrahepatic bile duct invasion ($p = 0.013$).

In 15 patients, a combination of intrahepatic lymphatic invasion and intrahepatic vascular invasion was present, and this combination was associated with a decreased OS (median 28.1 vs. 62.2 months) in univariate and multivariate analysis ($p<0.0001$).

Discussion

The current study describes the association between multiple histologic features in combination with clinical factors and survival in 124 patients who underwent liver resection for CRLM. A homogenous group of patients was evaluated because all patients underwent a complete resection (R0), for a solitary metastasis without neoadjuvant systemic treatment. The only significant histologic factor associated with decreased survival in multivariate analysis was presence of intrahepatic lymphatic invasion, especially in combination with intrahepatic vascular invasion.

Other authors also described lymphatic invasion as a negative predictor for survival.[13, 18, 20] In the current study, we observed a relative high frequency of lymphatic invasion (26.6%) compared to earlier studies (12–15%).[18, 20] This might be due to the use of immunohistochemistry, which is supported by a recently published study with the same methodology and a similar frequency of lymphatic invasion (29%).[18, 20, 34-36] Presence of lymphatic invasion has been associated with spread to hepatic lymph nodes, which often leads to incurable disease.[20, 37] In the current study, the worse prognosis was demonstrated in patients with a combination of vascular and lymphatic invasion. This unfavorable combination has been observed before and might reflect a tumor with aggressive behavior.[23]

Another interesting finding from the current study was that the median tumor thickness at tumor–normal interface in patients who were not treated with neoadjuvant systemic therapy was 3.0 mm. This was only slightly higher than the tumor thickness of 2.8 mm described in patients treated with neoadjuvant chemotherapy.[26] This raises the question whether tumor thickness at tumor–normal interface reflects chemotherapy response or tumor biology; this would be an interesting subject for further research.

A major strength of the present study is the inclusion of patients with solitary CRLM only, who were operated with complete margins (R0) to create an homogenous group of patients. Previous studies on histologic prognostic factors included patients

with multiple CRLM and R1 resections as well, which might lead to significant bias of the results.[18, 20, 36] First, heterogeneity of histologic features between the different liver metastases might exist and could lead to bias studying prognostic factors for survival. Second, patients who undergo R1 resection usually have a higher risk of local recurrences and have an impaired survival.[38, 39] Third, patients with multiple metastases have a significantly decreased survival, and number of metastases is the most important factor in the Fong classification for survival.[9] By excluding these potential biases in the present study, the assessment of the prognostic histologic factors are more reliable.

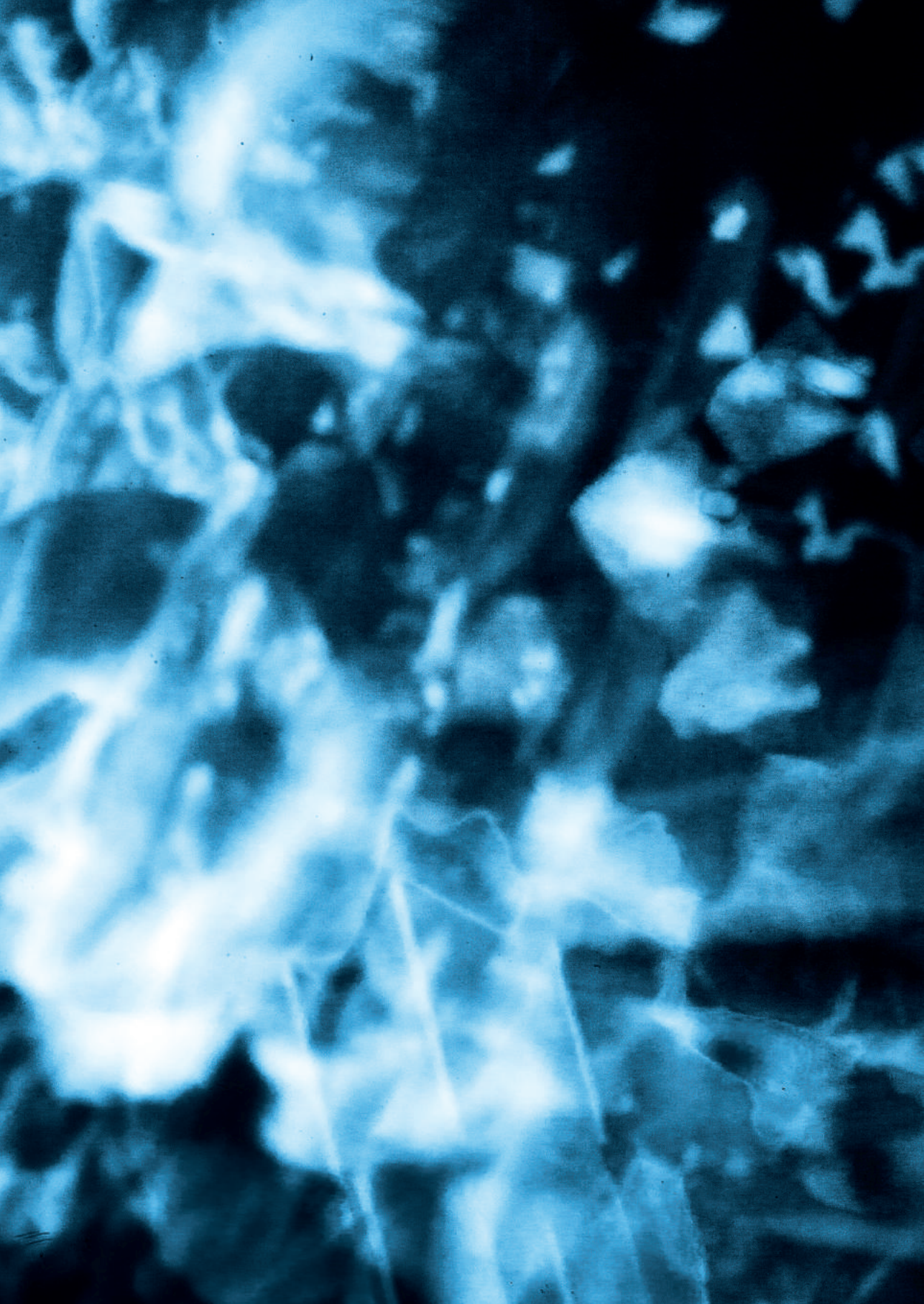
Another strength is that this homogenous group of patients with solitary metastasis were not treated with neoadjuvant systemic therapy. In recent studies, patients with and without neoadjuvant systemic therapy were mixed, and conclusions were drawn from a population highly susceptible to bias.[25, 36, 40] Neoadjuvant systemic therapy has a significant impact on tumor histology, and even prognostic factors such as resection margins might be less important.[27, 28, 41] Because the detection of histologic prognostic factors in metastatic disease is still in its infancy and the effects of neoadjuvant systemic therapy on lymphatic invasion are unknown, a study with an homogeneous population should be a first step. However, there seems to be an increasing preference to utilize neoadjuvant systemic therapy for high risk patients, despite a lack of convincing evidence on survival benefit in patients with limited metastases.[42-44] Therefore, a limitation of the present study is that the impact of lymphatic invasion on survival has to be confirmed in patients treated with neoadjuvant systemic therapy. In the total group of patients treated in our institution only 5 patients (3.8%) with solitary metastasis were treated with neoadjuvant chemotherapy, which made it impossible to compare, but this should be the goal for future research.

In conclusion, intrahepatic lymphatic invasion, based on immunohistochemical detection of lymphatic vessels, is an adverse prognostic factor for OS in patients with a solitary CRLM. Therefore, we recommend evaluating the presence or absence of intrahepatic lymphatic and vascular invasion in the histologic assessment of CRLM. Future research is needed to determine whether adjuvant treatment strategies should be based on these adverse prognostic histologic factors.

References

1. Jemal, A., et al., *Global cancer statistics.* CA Cancer J Clin, 2011. **61**(2): p. 69-90.
2. Zakaria, S., et al., *Hepatic resection for colorectal metastases: value for risk scoring systems?* Annals of surgery, 2007. **246**(2): p. 183-91.
3. House, M.G., et al., *Survival after hepatic resection for metastatic colorectal cancer: trends in outcomes for 1,600 patients during two decades at a single institution.* J Am Coll Surg, 2010. **210**(5): p. 744-52, 752-5.
4. Choti, M.A., et al., *Trends in long-term survival following liver resection for hepatic colorectal metastases.* Ann Surg, 2002. **235**(6): p. 759-66.
5. Hayashi, M., et al., *Clinicopathological analysis of recurrence patterns and prognostic factors for survival after hepatectomy for colorectal liver metastasis.* BMC Surg, 2010. **10**: p. 27.
6. Iwatsuki, S., et al., *Hepatic resection for metastatic colorectal adenocarcinoma: a proposal of a prognostic scoring system.* J Am Coll Surg, 1999. **189**(3): p. 291-9.
7. Konopke, R., et al., *Prognostic factors and evaluation of a clinical score for predicting survival after resection of colorectal liver metastases.* Liver Int, 2009. **29**(1): p. 89-102.
8. Nagashima, I., et al., *Proposal of criteria to select candidates with colorectal liver metastases for hepatic resection: Comparison of our scoring system to the positive number of risk factors.* World Journal of Gastroenterology, 2006. **12**(39): p. 6305-6309.
9. Fong, Y., et al., *Clinical score for predicting recurrence after hepatic resection for metastatic colorectal cancer: analysis of 1001 consecutive cases.* Ann Surg, 1999. **230**(3): p. 309-18; discussion 318-21.
10. Krasna, M.J., et al., *Vascular and neural invasion in colorectal carcinoma. Incidence and prognostic significance.* Cancer, 1988. **61**(5): p. 1018-23.
11. Shirouzu, K., et al., *A prospective clinicopathologic study of venous invasion in colorectal cancer.* Am J Surg, 1991. **162**(3): p. 216-22.
12. Benson, A.B., et al., *American society of clinical oncology recommendations on adjuvant chemotherapy for stage II colon cancer.* Journal of Clinical Oncology, 2004. **22**(16): p. 3408-3419.
13. Knijn, N., et al., *Histopathological evaluation of resected colorectal cancer liver metastases: what should be done?* Histopathology, 2013. **63**(2): p. 149-56.
14. Yamamoto, J., et al., *Factors influencing survival of patients undergoing hepatectomy for colorectal metastases.* Br J Surg, 1999. **86**(3): p. 332-7.
15. Yamamoto, J., et al., *Pathologic support for limited hepatectomy in the treatment of liver metastases from colorectal cancer.* Ann Surg, 1995. **221**(1): p. 74-8.
16. Tanaka, K., et al., *Effectiveness of prehepatectomy intra-arterial chemotherapy for multiple bilobar colorectal cancer metastases to the liver: A clinicopathologic study of peritumoral vasculobiliary invasion.* Surgery, 2005. **137**(2): p. 156-164.
17. Shirabe, K., et al., *Analysis of prognostic risk factors in hepatic resection for metastatic colorectal carcinoma with special reference to the surgical margin.* Br J Surg, 1997. **84**(8): p. 1077-80.
18. Sasaki, A., et al., *Prognostic significance of intrahepatic lymphatic invasion in patients with hepatic resection due to metastases from colorectal carcinoma.* Cancer, 2002. **95**(1): p. 105-11.
19. Okano, K., et al., *Macroscopic intrabiliary growth of liver metastases from colorectal cancer.* Surgery, 1999. **126**(5): p. 829-34.
20. Korita, P.V., et al., *Intrahepatic lymphatic invasion independently predicts poor survival and recurrences after hepatectomy in patients with colorectal carcinoma liver metastases.* Annals of Surgical Oncology, 2007. **14**(12): p. 3472-3480.
21. Okano, K., et al., *Lymphocytic infiltration surrounding liver metastases from colorectal cancer.* Journal of Surgical Oncology, 2003. **82**(1): p. 28-33.
22. Kubo, M., et al., *Less aggressive features of colorectal cancer with liver metastases showing macroscopic intrabiliary extension.* Pathol Int, 2002. **52**(8): p. 514-518.

23. Bockhorn, M., et al., *Prognostic impact of intrahepatic lymphatic and microvascular involvement in cases of colorectal liver metastases.* Int J Colorectal Dis, 2009. **24**(7): p. 845-850.
24. Okano, K., et al., *Fibrous pseudocapsule of metastatic liver tumors from colorectal carcinoma. Clinicopathologic study of 152 first resection cases.* Cancer, 2000. **89**(2): p. 267-275.
25. Brunner, S.M., et al., *Prognosis according to histochemical analysis of liver metastases removed at liver resection.* Br J Surg, 2014. **101**(13): p. 1681-91.
26. Maru, D.M., et al., *Tumor thickness at the tumor-normal interface: a novel pathologic indicator of chemotherapy response in hepatic colorectal metastases.* Am J Surg Pathol, 2010. **34**(9): p. 1287-1294.
27. Gervaz, P., et al., *Neoadjuvant chemotherapy in patients with stage IV colorectal cancer: a comparison of histological response in liver metastases, primary tumors, and regional lymph nodes.* Ann Surg Oncol, 2010. **17**(10): p. 2714-9.
28. van der Pool, A.E., et al., *Effect of bevacizumab added preoperatively to oxaliplatin on liver injury and complications after resection of colorectal liver metastases.* J Surg Oncol, 2012. **106**(7): p. 892-7.
29. Loupakis, F., et al., *Histopathologic evaluation of liver metastases from colorectal cancer in patients treated with FOLFOXIRI plus bevacizumab.* Br J Cancer, 2013. **108**(12): p. 2549-56.
30. Halama, N., et al., *Hepatic metastases of colorectal cancer are rather homogeneous but differ from primary lesions in terms of immune cell infiltration.* Oncoimmunology, 2013. **2**(4): p. e24116.
31. Abengozar, M., et al., *Prognostic utility of tumor thickness at the tumor-normal interface in chemotherapy-treated hepatic colorectal metastasis.* Pathol Res Pract, 2012. **208**(4): p. 235-9.
32. Brouquet, A., et al., *Multicenter validation study of pathologic response and tumor thickness at the tumor-normal liver interface as independent predictors of disease-free survival after preoperative chemotherapy and surgery for colorectal liver metastases.* Cancer, 2013. **119**(15): p. 2778-88.
33. Vlems, F., et al., *A study into methodology and application of quantification of tumour vasculature in rectal cancer.* Virchows Archiv, 2004. **445**(3): p. 263-270.
34. Mohammed, R.A.A., et al., *Improved methods of detection of lymphovascular invasion demonstrate that it is the predominant method of vascular invasion in breast cancer and has important clinical consequences.* American Journal of Surgical Pathology, 2007. **31**(12): p. 1825-1833.
35. Van den Eynden, G.G., et al., *Distinguishing blood and lymph vessel invasion in breast cancer: a prospective immunohistochemical study.* British Journal of Cancer, 2006. **94**(11): p. 1643-1649.
36. Lupinacci, R.M., et al., *Intrahepatic Lymphatic Invasion but not Vascular Invasion is a Major Prognostic Factor after Resection of Colorectal Cancer Liver Metastases.* World Journal of Surgery, 2014. **38**(8): p. 2089-2096.
37. August, D.A., P.H. Sugarbaker, and P.D. Schneider, *Lymphatic dissemination of hepatic metastases. Implications for the follow-up and treatment of patients with colorectal cancer.* Cancer, 1985. **55**(7): p. 1490-4.
38. Angelsen, J.H., et al., *Surgery for colorectal liver metastases: the impact of resection margins on recurrence and overall survival.* World Journal of Surgical Oncology, 2014. **12**.
39. Vigano, L., et al., *Early recurrence after liver resection for colorectal metastases: risk factors, prognosis, and treatment. A LiverMetSurvey-based study of 6,025 patients.* Ann Surg Oncol, 2014. **21**(4): p. 1276-86.
40. John, S.K., et al., *Prognostic factors and survival after resection of colorectal liver metastasis in the era of preoperative chemotherapy: an 11-year single-centre study.* Dig Surg, 2013. **30**(4-6): p. 293-301.
41. Ayez, N., et al., *Outcome of microscopic incomplete resection (R1) of colorectal liver metastases in the era of neoadjuvant chemotherapy.* Ann Surg Oncol, 2012. **19**(5): p. 1618-27.
42. Nathan, H., et al., *Treating patients with colon cancer liver metastasis: a nationwide analysis of therapeutic decision making.* Ann Surg Oncol, 2012. **19**(12): p. 3668-76.
43. Vauthey, J.N., et al., *RAS mutation status predicts survival and patterns of recurrence in patients undergoing hepatectomy for colorectal liver metastases.* Ann Surg, 2013. **258**(4): p. 619-26; discussion 626-7.
44. Nordlinger, B., et al., *Perioperative FOLFOX4 chemotherapy and surgery versus surgery alone for resectable liver metastases from colorectal cancer (EORTC 40983): long-term results of a randomised, controlled, phase 3 trial.* Lancet Oncol, 2013. **14**(12): p. 1208-15.



Chapter 3

KRAS mutation analysis: a comparison between primary tumours and matched liver metastases in 305 colorectal cancer patients

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Abstract

KRAS mutation is a negative predictive factor for treatment with anti-epidermal growth factor receptor antibody in metastatic colorectal cancer (CRC). *KRAS* mutation analysis is usually performed on primary tumour tissue because metastatic tissue is often not available. However, controversial data are available on the concordance of test results between primary tumours and corresponding metastases. We assessed the concordance of *KRAS* mutation status in a study of 305 primary colorectal tumours and their corresponding liver metastases.

Patients with histologically confirmed CRC who underwent surgical resection of the primary tumour and biopsy or surgical resection of the corresponding liver metastasis were included. *KRAS* mutation analysis was performed for codons 12 and 13.

KRAS mutation was detected in 108 out of 305 primary tumours (35.4%). In 11 cases (3.6%), we found a discordance between primary tumour and metastasis: 5 primary tumours had a *KRAS* mutation with a wild-type metastasis, 1 primary tumour was wild type with a *KRAS* mutation in the metastasis, and in 5 cases the primary tumour and the metastasis had a different *KRAS* mutation.

We observed a high concordance of *KRAS* mutation status of 96.4% (95% CI 93.6–98.2%) between primary colorectal tumours and their corresponding liver metastases. In only six patients (2.0%; 95% CI 0.7–4.2%), the discordance was clinically relevant. In this largest and most homogenous study to date, we conclude that both primary tumours and liver metastases can be used for *KRAS* mutation analysis.

Introduction

Recent advances in specific signalling pathways of cancer cells have introduced targeted therapy into treatment regimes for patients with metastatic colorectal cancer (CRC).[1] Cetuximab and panitumumab are monoclonal antibodies that bind to the extracellular domain of the epidermal growth factor receptor (EGFR). They inhibit ligand-induced stimulation of several intracellular signalling pathways, such as RAS/RAF/MAPK and phosphoinositide-3 pathway, which results in decreased stimulation of cell cycle progression, proliferation, angiogenesis, and stimulation of apoptosis.[2] The *KRAS* oncogene is currently the most relevant molecular biomarker that predicts the response to EGFR-targeted therapy in CRC. An oncogenic mutation in *KRAS* leads to constitutive activation of the RAS/RAF signalling pathway independent from EGFR activation by binding of the ligand.[3]

KRAS mutations occur in approximately 38% of colorectal tumours and involve codon 12 and 13 in 496% of cases.[4] Metastatic CRC patients with tumours harbouring a *KRAS* mutation are resistant to treatment with anti-EGFR antibodies, showing lower response rates, decreased progression- free survival, and overall survival compared with patients with *KRAS* wild-type tumours.[5-7] Therefore, the European Medicines Agency and the Food and Drug Administration have restricted the use of anti-EGFR antibodies in metastatic CRC to patients with *KRAS* wild-type tumours.

Cetuximab and panitumumab have shown efficacy both as monotherapy[5, 8] and in combination with chemotherapy[6, 7] in patients with *KRAS* wild-type metastatic CRC. Nevertheless, even among patients with *KRAS* wild-type tumours, the majority of patients do not respond to anti-EGFR therapy. Efficacy of anti-EGFR therapy was suggested to be further restricted to patients with *BRAF* wild-type tumours.[9] An additional explanation for the suboptimal response rates to anti-EGFR antibodies in patients with *KRAS* wild-type tumours is discordance of *KRAS* mutation status between primary colorectal tumours and corresponding metastases. In the early dissemination model, tumour cells depart the primary lesion before the acquisition of a fully malignant phenotype to undergo new mutations and metastatic growth at a distant site.[10] According to this model, a discordance in mutation status between primary tumours and metastases may occur, and as a consequence the mutation status of the primary tumour might not be adequate to predict the response of metastases to anti-EGFR treatment. Current data on the concordance in *KRAS* mutation status between primary colorectal tumours and metastases are conflicting.

Five studies showed a 100% concordance of *KRAS* mutation status in primary CRC and corresponding metastases.[11-15] In contrast to these data, others have reported a discordance of *KRAS* mutation status in primary tumours and metastatic sites, with

an overall discordance observed in 4–32% of the patients.[16-28] These controversial results are probably due to the fact that these studies were underpowered with a small number of patients, and included a wide variety of metastatic sites. Therefore, it is still uncertain whether the evaluation of *KRAS* mutation status in the most commonly available primary tumour correctly reflects the *KRAS* mutation status of corresponding metastasis. This is highly relevant given the large number of CRC patients as well as the potential toxicity and costs of anti-EGFR therapy. We assessed the concordance in *KRAS* mutation status in primary tumours and their corresponding liver metastases in an adequately powered study of 305 CRC patients.

Material and methods

Patient selection

Patients with histologically confirmed CRC who underwent surgical resection of the primary tumour and biopsy or surgical resection of the corresponding liver metastasis were included in this analysis. Results were obtained from archived material of three large pathology laboratories and from material collected from the CAIRO2 study, a large multicentre trial of the Dutch Colorectal Cancer Group.[6]

In patients with a discordance of *KRAS* mutation status between the primary tumour and metastasis, additional blocks of the primary tumour were obtained to exclude heterogeneity within the tumour. Lymph node metastases present at the time of diagnosis were also acquired in these patients.

Tumour DNA preparation

Formalin-fixed paraffin-embedded tissue blocks were cut at 4 mm thickness and stained with haematoxylin and eosin (HE). The presence of tumour tissue was marked by a pathologist. Subsequently the blocks were cut at 20 – 40 mm thickness and micro dissected for DNA extraction. Tumour tissue was dissolved in 200 ml lysis buffer (QIAamp DNA Micro Kit, Qiagen, Venlo, The Netherlands) and incubated with proteinase K overnight at 56 1C for two nights. DNA was extracted according to the manufacturer's protocol (QIAamp DNA Micro Kit, Qiagen), and DNA concentration was determined at 260 nm using the Nanodrop 26 ND-1000 spectro- photometer (Nanodrop Technologies Inc., Wilmington, NC, USA).

KRAS mutation analysis

For *KRAS* mutation analysis, exon 2 (codon 12 and 13) was amplified using a 50 ml reaction mixture containing 0.2 mm forward (5′-TGTAAAACGACGGCCAGTAGGCCTGCTGAAAATG ACTG-3′) and reverse (5′-CAGGAAACAGCTATGACCTGGATCATATTCGTCCACAAAA-3′) primers (Invitrogen, Breda, The Netherlands); dATP, dCTP, dGTP and dTTP (GE Healthcare, Zeist, The Netherlands) at 0.2 mM each; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 2.5 mM MgCl2; 1 U AmpliTaq Gold polymerase (Applied Biosystems, Nieuwkerk a/d IJsel, The Netherlands) and 50 ng of template DNA. The PCR conditions were as follows: 94 1C for 10 min; 92 1C for 1 min, 60 1C for 1 min, 72 1C for 1 min (40 cycles); and 72 1C for 10 min.

All PCR products were purified with the MultiScreen HTS, 96 well Filtration System (Millipore, Carrigtwohill, Ireland). Subsequently, the purified products were sequenced using fluorescently labelled terminators (BigDye Terminators (v 1.1); Applied Biosystems, Foster City, CA, USA) with both M13-forward and M13-reverse sequencing primers. The sequencing products were analysed on an ABI 3730 DNA Analyser (Applied Biosystems) and the data analysis was performed using Sequencing Analysis Software Sequencing Analysis Software v5.3.1 with KBTM Base- caller. Sequence results were scored by visual inspection of the chromatograms (Applied Biosystems).

Statistical analysis

We considered a discordance level of 5% or more to be clinically relevant, that is, leading to substantial change in routine clinical practice. To exclude such level of discordance under the assumption that the true discordance was 2.5% or less, we set the sample size at 304 paired samples. With this sample size, the precision in the estimated percentage of discordance was 2.5% (i.e., s.e. 1.25, half- width of the 95% confidence interval equal to 2.5%).

The comparison of patient and primary tumour characteristics between patients with *KRAS* wild-type and *KRAS* mutant primary tumours was done using Wilcoxon's rank sum test or χ^2 for numerical or categorical variables, respectively. Differences in *KRAS* mutation status between the primary tumour and corre- sponding metastasis were analysed by calculating the percentage of concordance, and (clinically relevant) discordance, together with the corresponding Clopper– Pearson 95% confidence intervals. Differences were considered to be statistically significant when the *P*-value was below 0.05. All statistical tests were two-sided.

Results

Patient characteristics

We analysed *KRAS* codon 12 and 13 mutations in 320 matched primary colorectal tumours and liver metastases. The tumour cell percentages in all primary tumours and metastases were above 30%. We failed to obtain a *KRAS* mutation status in 15 patients; therefore our further analyses were performed in 305 paired samples. Patient characteristics are shown in Table 1.

KRAS mutation and histopathological parameters

A total of 108 patients (35.4%) had a *KRAS* mutation in the primary tumour; of which 37 patients had a Gly12Asp mutation, 28 patients a Gly12Val mutation, 14 patients a Gly13Asp mutation, 10 patients a Gly12Cys mutation, 7 patients a Gly12Ser mutation, 7 patients a Gly12Ala mutation, 3 patients a Gly12Arg mutation, 1 patient a Gly12Asp and Gly12Ala mutation and 1 patient a Gly12Phe mutation (Table 2). Histopathological characteristics of the primary tumour were comparable between patients with and without a *KRAS* mutation (Table 1).

Concordance of *KRAS* status in primary tumours and corresponding liver metastases

In 294 patients (96.4%; 95% CI 93.6–98.2%), the same *KRAS* mutation status was obtained from the primary tumour and the corresponding liver metastasis. In 11 patients (3.6%; 95% CI 1.8–6.4%), of which 7 had synchronous metastases at diagnosis and 4 developed metachronous metastases, we found a discordance between primary tumours and metastases. Five patients had a *KRAS* mutation in the primary tumour and not in the liver metastasis. Only one patient had a wild-type status of the primary tumour, while the metastasis showed a *KRAS* mutation. In five patients, the primary tumours had different *KRAS* mutations compared with the metastases. One of these patients had two primary tumours. Both primary tumours had the same *KRAS* mutation (Gly13Asp), while the liver metastasis had a different *KRAS* mutation (Gly12Ser). In another patient, the primary tumour had a double mutation (Gly12Asp/Gly12Val) and the metastasis had a Gly12Asp mutation (Figure 1, Table 3). Taken together, the observed discordance was clinically relevant in only six patients (2.0%; 95% CI 0.7–4.2%).

Table 1. Distribution of tumour characteristics according to *KRAS* status of the primary tumour

	Overall, n =305	*KRAS* mutation, n =108	*KRAS* wild type, n = 197	P-value
Age				0.20
Median (IQR)	64 (57 – 70)	65 (58 – 71)	64 (57 – 70)	
Gender				0.37
Male	191 (62.6%)	64 (59.3%)	127 (64.5%)	
Female	114 (37.4%)	44 (40.7%)	70 (35.5%)	
Metastases presentation				0.45
Synchronous	169 (55.4%)	63 (58.3%)	106 (53.8%)	
Metachronous	136 (44.6%)	45 (41.7%)	91 (46.2%)	
Tumour location				0.63
Colon	167 (54.8%)	59 (54.6%)	108 (54.8%)	
Rectum	54 (17.7%)	16 (14.8%)	38 (19.3%)	
Rectosigmoid	80 (26.2%)	32 (29.6%)	48 (24.4%)	
Unknown	4 (1.3%)	1 (0.9%)	3 (1.5%)	
Histopathological subtype				0.12
Adenocarcinoma	271 (88.9%)	90 (83.3%)	181 (91.9%)	
Adenocarcinoma with muc. component	21 (6.9%)	10 (9.3%)	11 (5.6%)	
Mucinous adenocarcinoma	8 (2.6%)	5 (4.6%)	3 (1.5%)	
Unknown	5 (1.6%)	3 (2.8%)	2 (1.0%)	
Differentiation grade				0.21
Good	33 (10.8%)	13 (12.0%)	20 (10.2%)	
Moderate	196 (64.3%)	65 (60.2%)	131 (66.5%)	
Poor	52 (17.0%)	17 (15.7%)	35 (17.8%)	
Unknown	24 (7.9%)	13 (12.0%)	11 (5.6%)	
T stage				0.62
T1	4 (1.3%)	2 (1.9%)	2 (1.0%)	
T2	20 (6.6%)	9 (8.3%)	11 (5.6%)	
T3	231 (75.7%)	81 (75.0%)	150 (76.1%)	
T4	36 (11.8%)	11 (10.2%)	25 (12.7%)	
Unknown	14 (4.6%)	5 (4.6%)	9 (4.6%)	
N stage				0.10
N0	114 (37.4%)	46 (42.6%)	68 (34.5%)	
N1	87 (28.5%)	31 (28.7%)	56 (28.4%)	
N2	86 (28.2%)	26 (24.1%)	60 (30.5%)	
Unknown	18 (5.9%)	5 (4.6%)	13 (6.6%)	
Number of lymph nodes examined				0.28
Median (IQR)	10 (6 – 15)	10 (6 – 13)	10 (6 – 16)	
Number of lymph node metastases				0.15
Median (IQR)	1 (0 – 4)	1 (0 – 3)	1 (0 – 4)	

Abbreviation: IQR = interquartile range

Subsequent analyses in patients with a discordance of *KRAS* status

Several tests were performed to exclude bias of the test results. First, the HE coupes of all patients with a discordant *KRAS* mutation status between the primary tumour and liver metastasis were revised. The primary tumours and liver metastases had a mean tumour cell percentage of 65 and 60%, respectively. Subsequent independent reanalysis of the *KRAS* mutation status resulted in the same discordances.

Second, several mutation analyses were performed on different areas of the tumour and from different tumour blocks in order to establish possible tumour heterogeneity. Two patients showed heterogeneity of *KRAS* status within the primary tumour. One of these patients demonstrated two areas with a Gly12Asp mutation and one area with wild-type status, of which the latter resembled the liver metastasis. The other patient showed two different *KRAS* mutations within the same tumour, of which one is concordant with the liver metastasis (Table 3).

Third, 6 of the 11 patients with discordant results did have lymph nodes metastases at the time of diagnosis. *KRAS* mutation testing of all lymph nodes separately revealed overall concordant *KRAS* status between lymph node metastases and the primary tumour in three patients. The *KRAS* status of the lymph nodes in the other three patients showed heterogeneity, of which at least one lymph node metastases showed a different *KRAS* status compared with the primary tumour. However, this explains the discordance between the primary tumour and liver metastasis only in one patient (Table 3).

Discussion

This is the first adequately powered study in CRC that compares *KRAS* mutation status between primary tumours and their corresponding liver metastases. We showed that tissue from the primary tumour can reliably be used for *KRAS* mutation testing in order to select patients for anti-EGFR therapy.

We observed a concordant *KRAS* mutation status in 96.4% of 305 paired samples of colorectal tumours and liver metastases. However, the difference in *KRAS* status was not clinically relevant in 5 of the 11 patients with discordant results, because both primary tumour and metastasis had a different *KRAS* mutation. Given the high statistical power of our analysis, we were able to obtain a highly accurate estimate of the level of discordance that enabled us to conclude that the level of discordance was 2.0%. The high

Table 2. Distribution of *KRAS* mutation types

Codon 12/13	Patients with *KRAS* mutation (n, %)
Gly12Asp	37 (34%)
Gly12Val	28 (26%)
Gly13Asp	14 (13%)
Gly12Cys	10 (9%)
Gly12Ser	7 (6%)
Gly12Ala	7 (6%)
Gly12Arg	3 (3%)
Gly12Phe	1 (1%)
Gly12Asp + Gly12Val	1 (1%)

Table 3. Patients with a discordant *KRAS* status between primary tumour and liver metastasis. Multiple blocks of primary tumour tissue and lymph node metastases were tested when available**.**

	KRAS status primary tumour	*KRAS* status 2nd tumour	*KRAS* status LN metastasis	*KRAS* status liver metastasis
1	Gly12Ala	-	LN 1: Gly12Ala LN 2: Gly12Ala LN 3: Gly12Ala	WT
2	Gly12Asp Gly12Asp WT	-	-	WT
3	Gly12Cys	-	-	WT
4	Gly12Asp Gly12Asp Gly12Asp Gly12Asp	-	LN 1: Gly12Asp LN 2: Gly12Asp LN 3: Gly12Asp LN 4: Gly12Asp LN 5: WT	WT
5	Gly12Ser	-	-	WT
6	WT	-	-	Gly12Cys
7	Gly12Asp	-	LN 1: WT LN 2: WT LN 3: WT	Gly12Ala
8	Gly13Asp	Gly13Asp	LN 1: Gly13Asp	Gly12Ser
9	Gly12Ser	-	-	Gly12Ala
10	Gly12Cys Gly12Asp	-	LN 1: Gly12Asp LN 2: Gly12Asp LN 3: Gly12Asp LN 4: Gly12Asp LN 5: Gly12Asp LN 6: WT	Gly12Asp
11	Gly12Asp/Gly12Val	-	LN 1: Gly12Val LN 2: Gly12Val LN 3: Gly12Val LN 4: Gly12Asp LN 5: Gly12Asp LN 6: Gly12Asp LN 7: Gly12Asp	Gly12Asp

Abbreviation: WT = wild type

rate of concordance is in agreement with the notion that *KRAS* mutations are considered as early driving events in CRC progression, and associated with the growth of small adenoma to clinically significant size.[29] Therefore, *KRAS* mutation status is expected to be equal in both primary tumours and metastases.[10]

The previously reported lower concordance levels between primary tumours and metastases are most likely due to bias caused by false-negative results in underpowered studies. We calculated that 304 paired cases were needed to reliably exclude a rate of discordance of 45%, 110 patients (Table 4). Moreover, in these studies metastases of different sites were compared with the primary tumour. As the molecular patterns may differ between metastatic sites[10], more reliable results are obtained when *KRAS* mutation status is tested more rigorously for each metastatic site. The liver is the predominant site of metastases in the majority of metastatic CRC patients; therefore the results of our large series of 305 liver metastases provide a solid reference for clinical decision making as to anti-EGFR therapy. Another issue is the fact that *KRAS* testing is technically not as straightforward as is often assumed. Several quality assurance systems are now in place, and the first 'round robin' test indicates that at least 30% of the experienced pathology laboratories fail to pass the threshold level of the quality assurance programs.[30] Other important facts about *KRAS* testing are the correct evaluation of the amount of tumour tissue in the sample and the sensitivity of testing methods. In a previous study, we demonstrated in 4500 samples that both sequencing and real-time PCR are reliable methods.[31]

A discordant *KRAS* status between the primary tumour and metastasis was observed in a small number of patients (3.6%). In these cases, tumour cells may have departed the primary lesions before the acquisition of a fully malignant phenotype to undergo somatic mutations or deletions at a distant site.[10] Another explanation for the discordant results may be hetero- geneity of *KRAS* status within the primary tumour, although this was the case in only a small number of patients. Finally, a discordance may in theory be explained by metastases from a non- detected second primary.

Previously published data showed that a considerable fraction (25%, Table 4) of colorectal lymph node metastases does not resemble the primary tumour in terms of *KRAS* mutation status. In 5 of the 25 lymph node metastases that we tested the *KRAS* status was not concordant with the primary tumour, which is consistent with the literature (Table 4). Therefore, lymph node metastases do not seem suitable for determination of the *KRAS* mutation status of colorectal carcinomas. Discordance in *KRAS* mutation status might be due to clonal selection during the process of metastasis,

Table 4. Overview of studies providing data on *KRAS* status of primary tumour and related metastasis

Author study	Year	No. of pts	Analysed metastatic site	Method	*KRAS* mutation in PT (%)	*KRAS* mutation in PT, WT in M	*KRAS* WT in PT, mutation in M	Total percentage of discordance
Albanase	2004	30	Liver	SSCP analysis	14 (47%)	5/14 (36%)	4/16 (25%)	9/30 (30%)
Al-Mulla	1998	26	Liver	ASO/direct seq	10 (38%)	2/10 (20%)	3/16 (19%)	5/26 (19%)
		31	Lymph node	ASO/direct seq	10 (32%)	1/10 (10%)	5/21 (24%)	6/31 (19%)
Artale	2008	48	Diverse, 81% liver	Direct seq	11 (23%)	1/11 (9%)	2/37 (5%)	3/48 (6%)
Baldus	2010	20	Visceral metastasis	Direct seq	9 (45%)	1/9 (11%)	1/11 (9%)	2/20 (10%)
		55	Lymph node	Direct seq	29 (53%)	15/29 (52%)	2/26 (8%)	17/55 (31%)
Cejas	2010	93	Liver	Direct seq	30 (32%)	1/30 (3%)	4/63 (6%)	5/93 (5%)
		17	Lung	Direct seq	10 (59%)	1/10 (10%)	1/7 (14%)	2/17 (12%)
Etienne-Grimaldi	2008	48	Liver biopsy	PCR-RFLP	16 (33%)	0 (0%)	0 (0%)	0 (0%)
Italiano	2009	59	Not specified	Seq	23 (39%)	1/23 (4%)	2/36 (6%)	3/59 (5%)
Losi	1992	19	Local recurrence	Multiplex-ASPCR	12 (63%)	0 (0%)	0 (0%)	0 (0%)
		16	Metastasis, 38% liver	Multiplex-ASPCR	13 (81%)	0 (0%)	0 (0%)	0 (0%)
Loupakis	2009	43	Liver	Seq	Not mentioned	0 (0%)	2/*	2/43 (5%)
Molinari	2009	37	Diverse, 74% liver	Seq	16 (43%)	2/16 (13%)	1/21 (5%)	3/37 (8%)
		15	Lymph node	Seq	8 (53%)	0 (0%)	0 (0%)	0 (0%)
Oliveira	2006	28	Lymph node	Not mentioned	18 (64%)	2/18 (11%)	7/10 (70%)	9/28 (32%)
Oudejans	1991	31	Liver and lung	Hybridization	14 (45%)	1/14 (7%)	1/17 (6%)	2/31 (6%)
Perrone	2008	10	Diverse, mainly liver	Direct seq	2 (20%)	1/2 (50%)	1/8 (13%)	2/10 (20%)
Santini	2008	99	Diverse, 80% liver	Seq	38 (38%)	3/38 (8%)	1/61 (2%)	4/99 (4%)
Garm Spindler	2009	31	Not specified	qPCR	11 (35%)	2/11 (18%)	0/20 (0%)	2/31 (6%)
Suchy	1992	58	Autopsy material, not specified	Dot-blot hybridization	15 (26%)	0 (0%)	0 (0%)	0 (0%)
Weber	2006	36	Liver	Seq	14 (39%)	0 (0%)	0 (0%)	0 (0%)
Zauber	2003	42	Diverse, 93% lymph node, 5% liver	SCCP analysis + seq	22 (52%)	0 (0%)	0 (0%)	0 (0%)
Overall		892	All sites	All methods	345/849 (41%)	39/345 (11%)	35/504 (7%)	76/892 (9%)
		276	Liver	All methods	84/233 (36%)	8/84 (10%)	11/149 (7%)	21/276 (8%)
		129	**Lymph nodes**	**All methods**	**65/129 (50%)**	**18/65 (28%)**	**14/64 (22%)**	**32/129 (25%)**

Abbreviations: ASO=allele-specific oligonucleotide; ASPCR = allele-specific polymerase chain reaction; M metastasis; pts = patients; PT = primary tumour; qPCR = quantitative PCR; RFLP = restriction fragment length polymorphism; SSCP = single strand conformational polymorphism; seq = sequencing. *Total number of cases not specified.

however, heterogeneity in lymph node metastases could explain this discordance in only one patient.

Eight different *KRAS* mutation types were observed in our study, of which Gly12Asp showed the highest frequency. Five patients (1.6%) harboured different *KRAS* mutation types in the primary tumour compared with the metastases. This confirms the findings of Cejas *et al*[17] and Albanese *et al*[19], who reported a small number of patients (4 and 7%, respectively) with different mutation types between primary tumours and metastases. A different *KRAS* mutation type between primary lung adenocarcinomas and corresponding lymph node metastases was also observed in only 1% of the patients.[32] Currently, all patients with a *KRAS* mutation are excluded from treatment with anti-EGFR antibodies, independently of the mutation type. However, a recent paper indicated that codon 13 mutated tumours may be sensitive to cetuximab treatment.[33] As we observed a low frequency in *KRAS* mutation type discrepancies between primaries and metastases, this is not of clinical importance in selecting patients for anti-EGFR therapy.

In conclusion, we demonstrated a high level of concordance of 96.4% between primary tumours and liver metastases, which for clinical purposes to select CRC patients for anti-EGFR therapy was even higher with 98%. The implication of these results for general oncology practice is that both tissue of primary tumour or liver metastasis may be used for *KRAS* mutation testing. The results of our study are only valid for liver metastases and cannot be extrapolated to other metastatic locations. Furthermore, we demonstrated that discordance of test results between primary tumour and metastases cannot account for the failure rate of anti-EGFR therapy in patients with *KRAS* wild-type tumours. Therefore, novel predictive markers in addition to *KRAS* and *BRAF* mutation status are warranted.

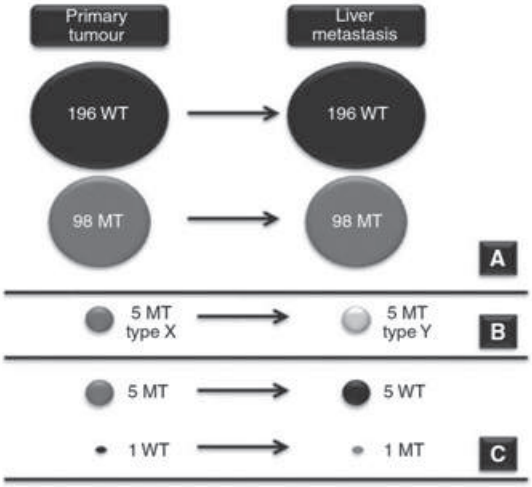

Figure 1. Overall concordance of the *KRAS* mutation status between primary tumour and liver metastasis (A), discordance without clinical impact (B), and discordance with clinical impact (C). Abbreviations: WT, wild type, MT, mutation.

References

1. Tol, J. and C.J. Punt, *Monoclonal antibodies in the treatment of metastatic colorectal cancer: a review.* Clin Ther, 2010. **32**(3): p. 437-53.
2. Scaltriti, M. and J. Baselga, *The epidermal growth factor receptor pathway: a model for targeted therapy.* Clin Cancer Res, 2006. **12**(18): p. 5268-72.
3. Benvenuti, S., et al., *Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies.* Cancer Res, 2007. **67**(6): p. 2643-8.
4. Oliveira, C., et al., *Distinct patterns of KRAS mutations in colorectal carcinomas according to germline mismatch repair defects and hMLH1 methylation status.* Hum Mol Genet, 2004. **13**(19): p. 2303-11.
5. Karapetis, C.S., et al., *K-ras mutations and benefit from cetuximab in advanced colorectal cancer.* N Engl J Med, 2008. **359**(17): p. 1757-65.
6. Tol, J., et al., *Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer.* N Engl J Med, 2009. **360**(6): p. 563-72.
7. Van Cutsem, E., et al., *Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer.* N Engl J Med, 2009. **360**(14): p. 1408-17.
8. Amado, R.G., et al., *Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer.* J Clin Oncol, 2008. **26**(10): p. 1626-34.
9. Di Nicolantonio, F., et al., *Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer.* J Clin Oncol, 2008. **26**(35): p. 5705-12.
10. Klein, C.A., *Parallel progression of primary tumours and metastases.* Nat Rev Cancer, 2009. **9**(4): p. 302-12.
11. Losi, L., J. Benhattar, and J. Costa, *Stability of K-ras mutations throughout the natural history of human colorectal cancer.* Eur J Cancer, 1992. **28A**(6-7): p. 1115-20.
12. Suchy, B., C. Zietz, and H.M. Rabes, *K-ras point mutations in human colorectal carcinomas: relation to aneuploidy and metastasis.* International journal of cancer, 1992. **52**(1): p. 30-3.
13. Zauber, P., et al., *Molecular changes in the Ki-ras and APC genes in primary colorectal carcinoma and synchronous metastases compared with the findings in accompanying adenomas.* Mol Pathol, 2003. **56**(3): p. 137-40.
14. Weber, J.C., et al., *Allelotyping analyses of synchronous primary and metastasis CIN colon cancers identified different subtypes.* Int J Cancer, 2007. **120**(3): p. 524-32.
15. Etienne-Grimaldi, M.C., et al., *K-Ras mutations and treatment outcome in colorectal cancer patients receiving exclusive fluoropyrimidine therapy.* Clin Cancer Res, 2008. **14**(15): p. 4830-5.
16. Oliveira, C., et al., *KRAS and BRAF oncogenic mutations in MSS colorectal carcinoma progression.* Oncogene, 2007. **26**(1): p. 158-63.
17. Cejas, P., et al., *KRAS mutations in primary colorectal cancer tumors and related metastases: a potential role in prediction of lung metastasis.* PLoS One, 2009. **4**(12): p. e8199.
18. Al-Mulla, F., et al., *Heterogeneity of mutant versus wild-type Ki-ras in primary and metastatic colorectal carcinomas, and association of codon-12 valine with early mortality.* J Pathol, 1998. **185**(2): p. 130-8.
19. Albanese, I., et al., *Heterogeneity within and between primary colorectal carcinomas and matched metastases as revealed by analysis of Ki-ras and p53 mutations.* Biochem Biophys Res Commun, 2004. **325**(3): p. 784-91.
20. Baldus, S.E., et al., *Prevalence and heterogeneity of KRAS, BRAF, and PIK3CA mutations in primary colorectal adenocarcinomas and their corresponding metastases.* Clin Cancer Res, 2010. **16**(3): p. 790-9.
21. Artale, S., et al., *Mutations of KRAS and BRAF in primary and matched metastatic sites of colorectal cancer.* J Clin Oncol, 2008. **26**(25): p. 4217-9.
22. Italiano, A., et al., *KRAS and BRAF mutational status in primary colorectal tumors and related metastatic sites: biological and clinical implications.* Ann Surg Oncol, 2010. **17**(5): p. 1429-34.

23. Loupakis, F., et al., *PTEN expression and KRAS mutations on primary tumors and metastases in the prediction of benefit from cetuximab plus irinotecan for patients with metastatic colorectal cancer.* J Clin Oncol, 2009. **27**(16): p. 2622-9.
24. Molinari, F., et al., *Differing deregulation of EGFR and downstream proteins in primary colorectal cancer and related metastatic sites may be clinically relevant.* Br J Cancer, 2009. **100**(7): p. 1087-94.
25. Perrone, F., et al., *PI3KCA/PTEN deregulation contributes to impaired responses to cetuximab in metastatic colorectal cancer patients.* Ann Oncol, 2009. **20**(1): p. 84-90.
26. Oudejans, J.J., et al., *Differential activation of ras genes by point mutation in human colon cancer with metastases to either lung or liver.* Int J Cancer, 1991. **49**(6): p. 875-9.
27. Santini, D., et al., *High concordance of KRAS status between primary colorectal tumors and related metastatic sites: implications for clinical practice.* Oncologist, 2008. **13**(12): p. 1270-5.
28. Garm Spindler, K.L., et al., *The importance of KRAS mutations and EGF61A>G polymorphism to the effect of cetuximab and irinotecan in metastatic colorectal cancer.* Ann Oncol, 2009. **20**(5): p. 879-84.
29. Vogelstein, B., et al., *Genetic alterations during colorectal-tumor development.* N Engl J Med, 1988. **319**(9): p. 525-32.
30. Bellon, E., et al., *External quality assessment for KRAS testing is needed: setup of a European program and report of the first joined regional quality assessment rounds.* Oncologist, 2011. **16**(4): p. 467-78.
31. Tol, J., et al., *Circulating tumour cells early predict progression-free and overall survival in advanced colorectal cancer patients treated with chemotherapy and targeted agents.* Ann Oncol, 2010. **21**(5): p. 1006-12.
32. Schmid, K., et al., *EGFR/KRAS/BRAF mutations in primary lung adenocarcinomas and corresponding locoregional lymph node metastases.* Clin Cancer Res, 2009. **15**(14): p. 4554-60.
33. De Roock, W., et al., *Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab.* JAMA, 2010. **304**(16): p. 1812-20.

Chapter 4

Sequencing of RAS/RAF pathway genes in primary colorectal cancer and matched liver and lung metastases

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Under submission

Abstract

Mutations in the RAS/RAF pathway predict resistance to anti-epidermal growth factor receptor antibodies in colorectal cancer (CRC), and may be targets for future therapies. This study investigates concordance of *BRAF*, *HRAS*, *KRAS*, *NRAS* and *PIK3CA* mutation status in primary CRC with matched liver (n=274), lung (n=114) or combined liver and lung metastases (n=14).

Next generation sequencing was performed on DNA from formalin-fixed paraffin embedded CRC and matched liver and/or lung metastases, for recurrent mutations in *BRAF*, *HRAS*, *KRAS*, *NRAS* and *PIK3CA* and using the single-molecule molecular inversion probe method.

Paired sequencing results on all five genes were reached in 249 of the 402 cases (62%). The obtained number of unique reads was not always sufficient to confidently call the absence or presence of mutations for all regions of interest. The mutational status of matched pairs was highly concordant; 91.1% concordance for all five genes, 95.5% for *KRAS*, 99.1% for *NRAS*. Lung metastases more often harboured *RAS* mutations compared to liver metastases (71% vs. 48%, $p<0.001$).

In this large series of CRC we show that both primary tumors and corresponding metastases can be used to determine the mutational status for targeted therapy, given the high concordance rates. Next generation sequencing including single molecule tags is feasible, however in combination with archival formalin-fixed paraffin embedded material is limited by coverage depth.

Introduction

Monoclonal antibodies against the Epidermal Growth Factor receptor (EGFR) are nowadays firmly established within treatment regimens for patients with metastatic colorectal cancer (CRC). These antibodies inhibit ligand induced stimulation of several intracellular signalling pathways such as RAS/RAF/MAPK and phosphoinositide-3 (PI3K) pathway, which results in decreased stimulation of cell cycle progression, proliferation, angiogenesis and stimulation of apoptosis. The presence of activating mutations in the RAS/RAF signalling pathway limits the effects of this treatment.[1-3] It is therefore standard procedure to perform molecular testing in order to determine the indication for these types of therapy. However, whether to test the primary tumor or the metastasis remains a matter of debate.[4-6] In previous work we have shown that for a limited number of *KRAS* mutations there is minimal discordance between primary CRC and liver metastases[7] However, we performed conventional Sanger sequencing and did not test all relevant *RAS* genes, nor *BRAF* and *PIK3CA* genes.

Next generation sequencing is increasingly performed in daily clinical practice. One advantage over Sanger sequencing is that low-frequency mutations can be detected. However, application of enrichment methods to gain sufficient quantities of DNA may result in amplification bias. To overcome this issue single molecule tags can be used.[8, 9] Consequently, in the current study we applied a single-molecule molecular inversion probe (smMIP)-based next generation sequencing approach to investigate concordance rates for all relevant *BRAF*, *HRAS*, *KRAS*, *NRAS* and *PIK3CA* mutations in CRC with matched lung and liver metastases.

Material and methods

Patient selection

All patients with histologically confirmed CRC who underwent surgical resection of the primary tumor and a sufficient biopsy or surgical resection of the corresponding liver or lung metastasis between 1984 and 2011 were included in this analysis. In case of multiple primary tumors or metastases, all material was used for sequencing. Formalin-fixed-parafin-embedded (FFPE) material from three large pathology laboratories; Radboud university medical center, Nijmegen, Rijnstate hospital, Arnhem and Laboratory of Pathology East Netherlands, Hengelo, was used. The Institutional Review Board of the Radboud university medical center, Nijmegen, ruled that the current study does not require informed consent according to Dutch law, but based on national guidelines for the use of archival material, the Institutional Review Board agrees with the study proposal (CMO 2013/048, date 23/4/2013).

DNA extraction and mismatch repair status analysis

FFPE tissue blocks were cut at 4 µm thickness and stained with haematoxylin and eosin (HE). The slide with highest tumor cell percentage was selected and the presence of tumor was marked by an expert pathologist (IN). Samples with tumor cell percentages above 30% were included. Subsequently the blocks were cut at 20 – 40 mm thickness and macrodissected for DNA extraction. DNA was extracted according to the manufacturer's protocol (QIAamp DNA Micro Kit, Qiagen, Hilden, Germany), and DNA concentration was determined with Qubit (2.0, Life Technologies, Foster city, CA, USA). For each sample approximately 250 nanogram of DNA was included.

Mismatch repair status analysis was assessed by immunohistochemistry. Microsatellite instability (MSI) analysis was performed in all cases with absence or unequivocal protein expression, using five microsatellite markers (NR21, NR24, NR27, BAT 25 and BAT 26, pentaplex PCR system). A tumor was defined as MSI if at least two of the five markers showed instability.[10]

Mutational analysis smMIP sequencing

smMIP based sequencing was used to detect mutations in *BRAF* (exon 15, targeted codons D594-K601), *HRAS* (exon 2, targeted codons G12, G13, exon 3, targeted codons, A59, Q61), *KRAS* (exon 2, targeted codons G12, G13, exon 3, targeted codons A59, Q61, exon 4 targeted codons K117, A146), *NRAS* (exon 2, targeted codons G12, G13, exon 3, targeted codons A59, Q61, exon 4 targeted codons K117, A146), and *PIK3CA* (exon 10, targeted codons E542-Q546, exon 21, targeted codons M1043-G1049). This technique is clinically validated and implemented in the routine diagnostics workflow of our hospital and is extensively described elsewhere.[9] In short, smMIPs are long oligonucleotides consisting of two targeting arms, the extension probe and the ligation probe, joined by a backbone including a single molecule tag. The probe sequences are complementary to the sequences surrounding the target region. During the capture reaction, the smMIP mixture is hybridized to the DNA, followed by gapfilling through extension and ligation, resulting in a circular smMIP. Exonuclease treatment removes all linear DNA. The circular smMIP are amplified by PCR using barcoded primers recognizing sequences in the backbone of the smMIP. After target enrichment, products were pooled with comparable amounts of the smMIP enriched products (based on an agarose gel) and sequenced on a NextSeq500 instrument (Illumina, San Diego, CA, USA). The commercial analysis software Sequence Pilot (JSI medical systems, Ettenheim, Germany) was used for variant identification. Sequencing reads are aligned and reads sharing the same unique single molecule tag are merged into a consensus read sequence. This reduces the number of sequencing artifacts, mutations present in the genomic template are maintained. The settings as described

by Eijkelenboom et al[9] were used for generating the consensus reads and for variant calling. The transcripts for variant annotation were: *BRAF* ENST00000288602; *KRAS* ENST00000311936; *PIK3CA* ENST00000263967; *HRAS* ENST00000451590; *NRAS* ENST00000369535. After variant calling using the commercial software, all variants were manually inspected and curated based on Cosmic (Cosmic v.81 (May 2017), Sanger Institute) and Alamut (AlamutVisual 2.9.0 (Dec. 2016), Interactive Biosoftware). Furthermore, in the downstream analysis the minimum mutant allele frequency was set at 5%. In fact, 96% of the variants selected had an allele frequency greater than or equal to 10%. Therefore, the minimum absolute coverage to reliably exclude the presence of mutations was set at 125 combined. This threshold excludes, with an approximate certainty of >90%, the presence of a mutation at minimally 10% mutant allele frequency within the covered regions.

Statistical analysis

To compare patient and primary tumor characteristics between patients with wild-type and mutant sequencing results Wilcoxon's rank sum test or $\chi 2$ for numerical or categorical variables, respectively was used. Differences in mutation status between the primary tumor and corresponding metastasis were analyzed by calculating the percentage of concordance and discordance. Concordance was defined as both primary tumor and metastasis having no mutations (wildtype-wildtype) or exactly the same mutation (mutation-mutation, same variant). Discordance was defined as a mutation in either tumor or metastasis which was not found in the corresponding counterpart (wildtype-mutant, mutant-wildtype) or as two different mutations in tumor and metastasis (mutant-mutant, different variants). Statistical analyzes were performed using the statistical software package SPSS 20.0 (SPSS Inc, Chicago, Illinois, USA). Differences were considered to be statistically significant with a P-value below 0.05. All statistical tests were two-sided.

Results

A total of 402 patients with CRC were included, 274 with liver metastases, 114 with lung metastases and 14 with both liver and lung metastases. The majority of patients presented with a solitary liver metastasis (253 patients), 10 patients had multiple liver metastases (range 2-5), nine patients had two primary tumors together with one liver metastasis and two patients had two primaries and two liver metastases sequenced. In most patients with lung metastases (n=103) one metastasis was available for testing, nine patients had two lung metastases and two patients presented with two primaries and one lung metastasis.

Concordant cases

We observed an overall concordance rate of 91.1% (216 concordant/237 total) in mutation status for all five genes in paired samples. The individual concordance rates for *BRAF*, *HRAS*, *KRAS*, *NRAS* and *PIK3CA* were 99.6% (246 concordant/247 total), 100% (364 total), 95.5% (193 concordant/202 total), 99.1% (228 concordant/230 total), 94.7% (213 concordant/225 total), respectively. All discordant cases for *BRAF* and *NRAS* were based on mutations present in the primary tumor, as were the majority of the *KRAS* (6/7) and *PIK3CA* discordant cases (9/13) (Figure 1). The observed discordance in *RAS* was clinically relevant (i.e. would result in a different treatment approach) in 9 patients (3.9%).

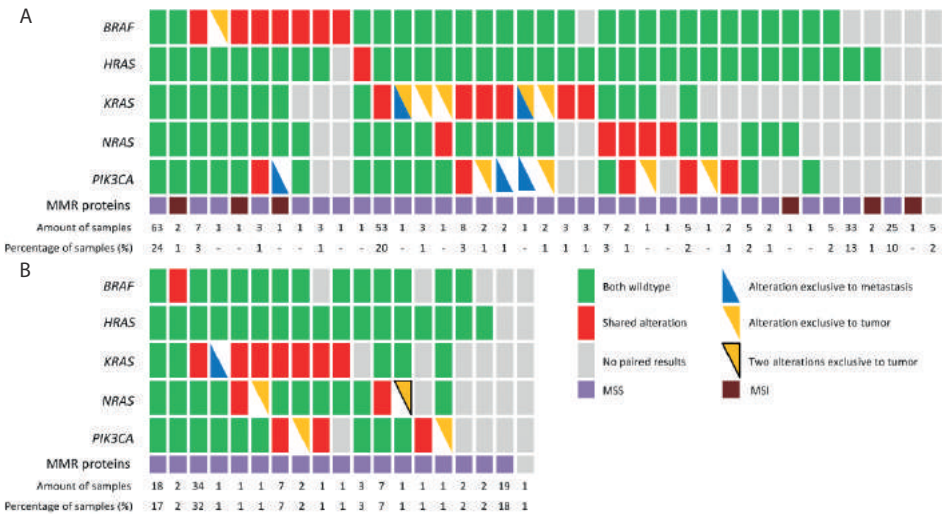


Figure 1. Overview of specific mutation and MMR status in primary tumors and matched single metastases. A: primary tumors with liver metastases; B: primary tumors with lung metastases.
Grey bars: insufficient unique reads to confidently classify the paired sample as wildtype or mutant (this could be due to insufficient reads of either the primary tumor, the metastasis or both).
The last two rows of the Figure depict the exact number of samples and the percentage of samples. A percentage less than 1% is depicted with a "·".
Abbreviations: MMR: mismatch repair; MSI: microsatellite instable; MSS: microsatellite stable.

Differences between liver and lung metastases

The overall mutation frequency in patients with lung metastases was higher than the mutation frequency in patients with liver metastases (Figure 2). The concordance rate in mutational status for all five genes was not statistically different for tumors with liver metastases and tumors with lung metastases (91.2% (166 concordant/182 total) vs. 89.1% (49 concordant/55 total), p=0.64). The rate of MSI was relatively low (2.2%), all patients with lung metastases were MSS and only eight patients with liver metastases showed MSI (3.2%).

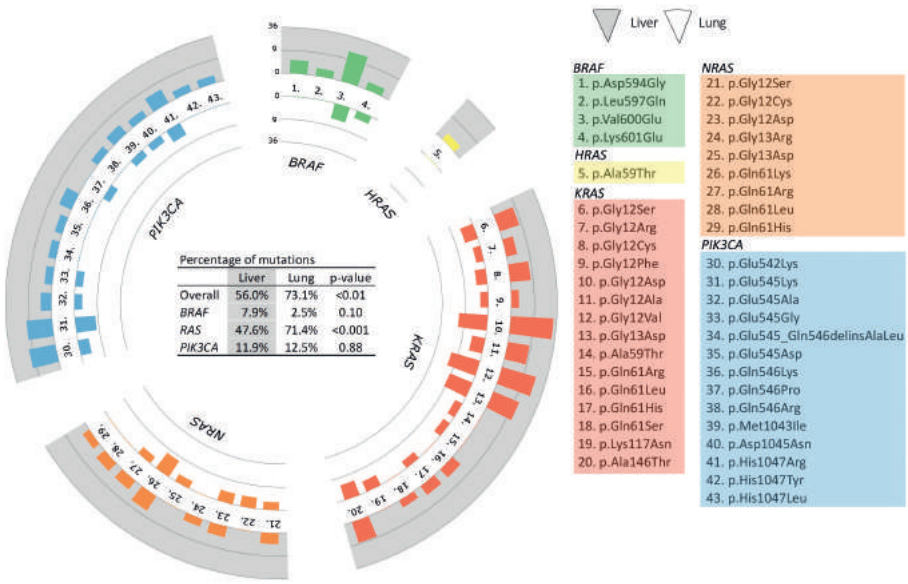


Figure 2. Overview of specific mutations found in patients with liver and lung metastases.
Mutations detected in liver metastases are shown in the grey panel, mutations detected in lung metastases are shown in white. An overall mutation frequency in percentages is given for different genes in the table. For specific mutations, the actual number of mutations is depicted.
*Patients with both liver and lung metastases are excluded.

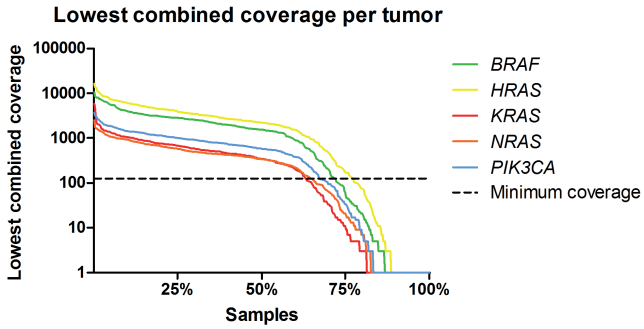
Multiple metastases

In 10 of the 14 patients with liver and lung metastases we obtained sequencing results of all tumor samples. In four patients we did not retrieve sufficient unique sequence reads (in three due to the primary tumor, in one due to failure of all samples). Identical results in mutation status of primary tumor, liver and lung metastases were observed in 9 of the 10 patients, (Figure 3A). In 10 of the 21 patients with multiple liver or lung metastases, sequencing results were obtained for both primary tumor and metastases. Insufficient sequence coverage

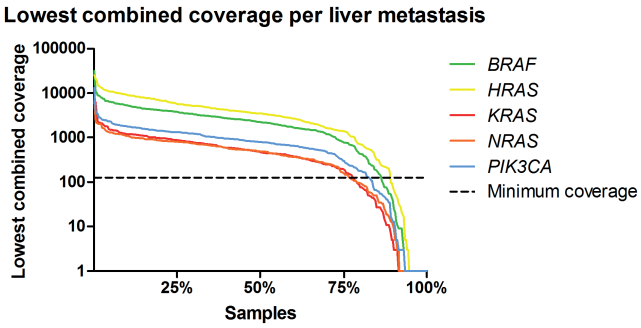
was due to failure of all samples (two patients), failure of primary tumor (six patients) or failure of metastases (three patients) 7 of the 10 patients showed concordance of primary tumor and metastases (Figure 3B, 3C). Discordance was due to mutations limited to the primary tumor (one case) and to the metastases (two cases).

Figure 3. Overview of mutations in primary tumors with multiple metastases. Data of samples with sufficient coverage on nearly all genes.
A: 10 primary tumors with both liver and lung metastases; 1 discordant patient with a *PIK3CA* alteration limited to the primary tumor.
B: 5 primary tumors with multiple liver metastases; 1 discordant patient with two different *KRAS* mutations in the primary tumor, of which one mutation was also detected in both metastases. This patient also had a *PIK3CA* alteration limited to the primary tumor.
C: 5 primary tumors with multiple lung metastases; 2 discordant patients; 1 patient with a *KRAS* alteration limited to both metastases, 1 patient with a *NRAS* mutation limited to one of the metastasis.
Grey bars: insufficient unique reads to confidently classify the multiple paired sample as wildtype or mutant (due to insufficient reads of one of the metastases).

A



B



C

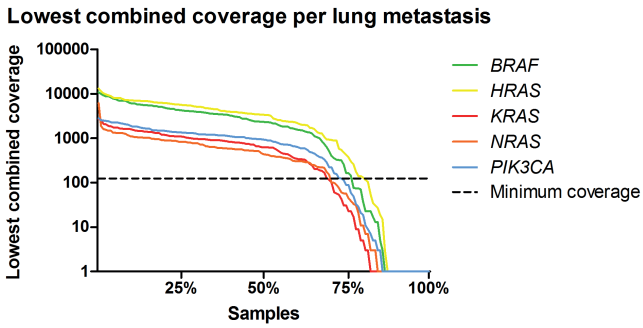


Figure 4. Lowest coverage per tissue type. 4A. Primary tumor, 4B. Liver metastasis, 4C. Lung metastasis. y-axis: the lowest coverage of samples in unique reads. Dotted line: the minimum absolute coverage of 125 unique reads x-axis: the percentage of samples.
In all samples *HRAS* mutation analysis performs better (coverage above 125 unique reads in the highest percentage of samples), followed by *BRAF*, *PIK3CA*, and *NRAS/KRAS*. Sequencing of liver metastases more often result in enough coverage (75% of samples), followed by lung metastases (70% of samples) and primary tumors (60% of samples).

Coverage

With our settings, we obtained paired sequencing results in 62.0% (249 concordant/402 total). The minimum coverage depth was reached for *BRAF*, *HRAS*, *KRAS*, *NRAS* and *PIK3CA* in 68%, 72%, 58%, 58% and 63% of the 415 primary tumors and in 80%, 83%, 68%, 70% and 74% of the 443 metastases (Figure 4). According to the type of material analyzed, metastases are more often complete on all five genes (69% of metastases vs. 59% of primary tumors, p=0.003).

Comparison with Sanger sequencing

In all patients *KRAS* Sanger sequencing was performed for exon 2. Good quality results of both Sanger and smMIP sequencing were available for 292 patients. In 81 patients results were only obtained with Sanger sequencing; coverage for the smMIP analysis was too low. In 29 patients results were only obtained with smMIP sequencing. When results from both techniques were available (n=292), concordance was 100%.

Discussion

This is the first large study that compares mutation status between primary tumors and their corresponding liver and lung metastases using a single molecule tag approach. We observed an overall concordance rate of 91% in all five genes in paired samples. Concordance rates above 99% were reached for *BRAF, HRAS* and *NRAS*, concordance for *KRAS* and *PIK3CA* were 96% and 95%, respectively. Comparable concordance rates for *KRAS* and *BRAF* are described in three smaller series.[11-13] In contrast, a discordance rate of 23% for *KRAS* and 7% for *BRAF* was detected in a study of 43 primary tumors and matched liver metastases.[14] Although an increased *KRAS* discordance rate was previously reported in CRC with lung metastases[15], we did not observe a difference in concordance rate between liver and lung metastases. These high concordance rates implicate that, in the treatment-naïve setting, there is no need for additional biopsies from metastatic sites in order to obtain a molecular profile to decide on anti-EGFR therapy. This is an important message, given the impact of additional interventions on patients, like shown in the meta-analysis of CT guided lung biopsies with overall complication rates of 24% to 38%.[16] Next to the increased costs and complications, the delay due to additional biopsies and subsequent testing might be considerable.

Mutation analysis based on the primary tumor, would have incorrectly withhold anti-EGFR treatment to 8 patients (3.4%) and one patient (0.5%) would have incorrectly received anti-EGFR therapy. Nevertheless, acquired resistance after anti-EGFR therapy, with novel *KRAS* or *NRAS* mutations in 38% to 60% of cases indicate the necessity of additional biopsies in that setting.[17, 18] [19]

We observed mutations in the RAS/RAF pathway in 62% of the metastases, ranging from 73% in lung metastases and 56% in liver metastases. The difference between mutation frequency in liver and lung metastases is mainly caused by the higher incidence of *RAS* mutations in lung metastases (71% vs. 47%). This high occurrence of *RAS* mutations in colorectal cancer with lung metastases is in line with literature.[15, 20, 21] *KRAS* mutations are also increased in colorectal bone and brain metastases.[22] Distinct metastatic patterns are observed for *BRAF* mutations as well, which are mainly associated with peritoneal and distant lymph node metastases.[23, 24] Furthermore, as expected,[25, 26] a low percentage of MSI was observed, and only in patients with liver metastases (3.2%). This confirms the inverse relation between MSI CRC and lung metastases.[24] MSI/*BRAF* mutated CRC seems to have a preference for widespread disease including peritoneal metastases, thus falling outside our current selection and explaining our relatively low incidence of MSI.

Our study illustrates the main limitation of next generation sequencing using single molecule tags, which is coverage dependency. This is even more apparent in our study because we need good coverage for all five genes in two or more samples per patient. In clinical setting limited coverage for an exon of a gene that is not directly involved in resistance to therapy, is less often a problem. However, this is potentially important since a test is only as strong as its lowest coverage. Sufficient unique sequence reads are required to reliably identify the presence or absence of a mutation. Adjusting the settings would increase the number of false positive and false negative calls. We have chosen a certainty level of >90% to reliably identify or exclude the presence of a mutation. Currently, there are no guidelines on what level of certainty is acceptable in the molecular setting. Since this has potentially major impact on important treatment decisions, it is vital that oncologists become aware of this issue. In our study the insufficient coverage was most likely a result of fragmented DNA due to FFPE fixation. Additional sequencing of the same cases did not result in a large improvement of coverage, probably because of this fragmented DNA. All archived material was obtained from a wide timeframe, ranging from 1984 to 2011. Although subanalysis did not show any correlation between year of resection and completeness of sequencing, older samples are believed to have more fragmented DNA.[27, 28]

In all cases with sufficient unique sequence reads, a high concordance of mutation status between primary tumors and metastases was observed. Therefore, we conclude that discordance in mutation status of anti-EGFR related genes is not an issue for molecular testing in treatment-naive CRC.

Table 1. Distribution of patient and tumor characteristics per mutation.

Patient/tumor characteristics	RAS		BRAF		PIK3CA	
	mut	wt	mut	wt	mut	wt
Age median (range)	64 (33-90)	63 (34-86)	63.5 (34-80)	63.5 (33-90)	63 (41-80)	63 (33-90)
Sex						
Male	106 (63)	113 (63)	12 (55)	198 (63)	21 (51)	183 (65)
Female	62 (37)	66 (37)	10 (45)	114 (37)	20 (49)	98 (35)
Location of tumor						
Colon	85 (51)	95 (53)	18 (82)	158 (51)	24 (59)	144 (51)
Rectum	39 (23)	46 (26)	3 (14)	75 (24)	6 (15)	71 (25)
Rectosigmoid	43 (26)	37 (21)	1 (5)	77 (25)	9 (22)	66 (23)
Unknown	1 (1)	1 (1)	-	2 (1)	2 (5)	-
Histology						
Adenocarcinoma	145 (86)	165 (92)	19 (86)	281 (90)	34 (83)	258 (92)
Adenocarcinoma with mucinous component	14 (8)	9 (5)	1 (5)	21 (7)	2 (5)	17 (6)
Mucinous adenocarcinoma	7 (4)	3 (2)	2 (9)	6 (2)	4 (10)	3 (1)
Unknown	2 (1)	2 (1)	-	4 (1)	1 (2)	3 (1)
T stage						
T1	2 (1)	3 (2)	0 (0)	5 (2)	1 (2)	4 (1)
T2	15 (9)	13 (7)	0 (0)	26 (8)	6 (15)	20 (7)
T3	133 (79)	140 (78)	18 (82)	245 (79)	27 (66)	225 (80)
T4	16 (10)	19 (11)	4 (18)	31 (10)	6 (15)	28 (10)
Unknown	2 (1)	4 (2)	-	5 (2)	1 (2)	4 (1)
N stage						
N0	75 (45)	66 (37)	5 (23)	132 (42)	17 (41)	116 (41)
N1	56 (33)	51 (28)	7 (32)	95 (30)	19 (46)	81 (29)
N2	36 (21)	54 (30)	10 (45)	77 (25)	5 (12)	76 (27)
Unknown	1 (1)	8 (4)	-	8 (3)	1 (2)	8 (3)
Location of metastases						
Liver	101 (60)	142 (79)	18 (82)	218 (70)	26 (64)	199 (71)
Lung	58 (35)	32 (18)	3 (14)	81 (26)	12 (29)	72 (26)
Both	9 (5)	5 (3)	1 (5)	13 (4)	3 (7)	10 (4)
Time to metastases						
Synchronous	64 (38)	84 (47)	12 (55)	130 (42)	18 (44)	116 (41)
Metachronous	104 (62)	95 (53)	10 (45)	182 (58)	23 (56)	165 (59)
MMR status						
MSS	163 (97)	168 (94)	19 (86)	302 (97)	41 (98)	269 (96)
MSI	1 (1)	8 (4)	3 (14)	3 (1)	1 (2)	5 (2)
No result	4 (2)	3 (2)	0 (0)	7 (2)	0 (0)	7 (2)

Abbreviations: *RAS: HRAS*, *NRAS* and *KRAS*; mut: mutant; wt: wildtype; MMR: Mismatch repair; MSS: microsatellite stable; MSI: microsatellite instable. Only samples reaching the acquired coverage per gene of interest are included in this table.

References

1. Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ, Zalcberg JR. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. The New England journal of medicine. 2008 Oct 23;359(17):1757-65.
2. Douillard JY, Oliner KS, Siena S, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, Rivera F, Kocakova I, Ruff P, Blasinska-Morawiec M, Smakal M, Canon JL, Rother M, Williams R, Rong A, Wiezorek J, Sidhu R, Patterson SD. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. The New England journal of medicine. 2013 Sep 12;369(11):1023-34.
3. Pietrantonio F, Petrelli F, Coinu A, Di Bartolomeo M, Borgonovo K, Maggi C, Cabiddu M, Iacovelli R, Bossi I, Lonati V, Ghilardi M, de Braud F, Barni S. Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: a meta-analysis. European journal of cancer. 2015 Mar;51(5):587-94.
4. Kim TM, Jung SH, An CH, Lee SH, Baek IP, Kim MS, Park SW, Rhee JK, Lee SH, Chung YJ. Subclonal Genomic Architectures of Primary and Metastatic Colorectal Cancer Based on Intratumoral Genetic Heterogeneity. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015 Oct 01;21(19):4461-72.
5. Sepulveda AR, Hamilton SR, Allegra CJ, Grody W, Cushman-Vokoun AM, Funkhouser WK, Kopetz SE, Lieu C, Lindor NM, Minsky BD, Monzon FA, Sargent DJ, Singh VM, Willis J, Clark J, Colasacco C, Rumble RB, Temple-Smolkin R, Ventura CB, Nowak JA. Molecular Biomarkers for the Evaluation of Colorectal Cancer: Guideline From the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology. The Journal of molecular diagnostics : JMD. 2017 Mar;19(2):187-225.
6. Tan C, Du X. KRAS mutation testing in metastatic colorectal cancer. World journal of gastroenterology : WJG. 2012 Oct 07;18(37):5171-80.
7. Knijn N, Mekenkamp LJ, Klomp M, Vink-Borger ME, Tol J, Teerenstra S, Meijer JW, Tebar M, Riemersma S, van Krieken JH, Punt CJ, Nagtegaal ID. KRAS mutation analysis: a comparison between primary tumours and matched liver metastases in 305 colorectal cancer patients. British journal of cancer. 2011 Mar 15;104(6):1020-6.
8. Hiatt JB, Pritchard CC, Salipante SJ, O'Roak BJ, Shendure J. Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. Genome research. 2013 May;23(5):843-54.
9. Eijkelenboom A, Kamping EJ, Kastner-van Raaij AW, Hendriks-Cornelissen SJ, Neveling K, Kuiper RP, Hoischen A, Nelen MR, Ligtenberg MJ, Tops BB. Reliable Next-Generation Sequencing of Formalin-Fixed, Paraffin-Embedded Tissue Using Single Molecule Tags. The Journal of molecular diagnostics : JMD. 2016 Nov;18(6):851-63.
10. Suraweera N, Duval A, Reperant M, Vaury C, Furlan D, Leroy K, Seruca R, Iacopetta B, Hamelin R. Evaluation of tumor microsatellite instability using five quasimonomorphic mononucleotide repeats and pentaplex PCR. Gastroenterology. 2002 Dec;123(6):1804-11.
11. Fujiyoshi K, Yamamoto G, Takahashi A, Arai Y, Yamada M, Kakuta M, Yamaguchi K, Akagi Y, Nishimura Y, Sakamoto H, Akagi K. High concordance rate of KRAS/BRAF mutations and MSI-H between primary colorectal cancer and corresponding metastases. Oncology reports. 2017 Feb;37(2):785-92.
12. Mao C, Wu XY, Yang ZY, Threapleton DE, Yuan JQ, Yu YY, Tang JL. Concordant analysis of KRAS, BRAF, PIK3CA mutations, and PTEN expression between primary colorectal cancer and matched metastases. Sci Rep. 2015 Feb 02;5:8065.
13. Cejas P, Lopez-Gomez M, Aguayo C, Madero R, Moreno-Rubio J, de Castro Carpeno J, Belda-Iniesta C, Barriuso J, Moreno Garcia V, Diaz E, Burgos E, Gonzalez-Baron M, Feliu J. Analysis of the concordance in the EGFR pathway status between primary tumors and related metastases of colorectal cancer patients:implications for cancer therapy. Curr Cancer Drug Targets. 2012 Feb;12(2):124-31.

14. Mostert B, Jiang Y, Sieuwerts AM, Wang H, Bolt-de Vries J, Biermann K, Kraan J, Lalmahomed Z, van Galen A, de Weerd V, van der Spoel P, Ramirez-Moreno R, Verhoef C, Ijzermans JN, Wang Y, Gratama JW, Foekens JA, Sleijfer S, Martens JW. KRAS and BRAF mutation status in circulating colorectal tumor cells and their correlation with primary and metastatic tumor tissue. International journal of cancer Journal international du cancer. 2013 Jul;133(1):130-41.
15. Kim MJ, Lee HS, Kim JH, Kim YJ, Kwon JH, Lee JO, Bang SM, Park KU, Kim DW, Kang SB, Kim JS, Lee JS, Lee KW. Different metastatic pattern according to the KRAS mutational status and site-specific discordance of KRAS status in patients with colorectal cancer. BMC cancer. 2012 Aug 09;12:347.
16. Heerink WJ, de Bock GH, de Jonge GJ, Groen HJ, Vliegenthart R, Oudkerk M. Complication rates of CT-guided transthoracic lung biopsy: meta-analysis. Eur Radiol. 2017 Jan;27(1):138-48.
17. Diaz LA, Jr., Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, Allen B, Bozic I, Reiter JG, Nowak MA, Kinzler KW, Oliner KS, Vogelstein B. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature. 2012 Jun 28;486(7404):537-40.
18. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, Valtorta E, Schiavo R, Buscarino M, Siravegna G, Bencardino K, Cercek A, Chen CT, Veronese S, Zanon C, Sartore-Bianchi A, Gambacorta M, Gallicchio M, Vakiani E, Boscaro V, Medico E, Weiser M, Siena S, Di Nicolantonio F, Solit D, Bardelli A. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 2012 Jun 28;486(7404):532-6.
19. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber B, Alani RM, Antonarakis ES, Azad NS, Bardelli A, Brem H, Cameron JL, Lee CC, Fecher LA, Gallia GL, Gibbs P, Le D, Giuntoli RL, Goggins M, Hogarty MD, Holdhoff M, Hong SM, Jiao Y, Juhl HH, Kim JJ, Siravegna G, Laheru DA, Lauricella C, Lim M, Lipson EJ, Marie SK, Netto GJ, Oliner KS, Olivi A, Olsson L, Riggins GJ, Sartore-Bianchi A, Schmidt K, Shih I M, Oba-Shinjo SM, Siena S, Theodorescu D, Tie J, Harkins TT, Veronese S, Wang TL, Weingart JD, Wolfgang CL, Wood LD, Xing D, Hruban RH, Wu J, Allen PJ, Schmidt CM, Choti MA, Velculescu VE, Kinzler KW, Vogelstein B, Papadopoulos N, Diaz LA, Jr. Detection of circulating tumor DNA in early- and late-stage human malignancies. Science translational medicine. 2014 Feb 19;6(224):224ra24.
20. Cejas P, Lopez-Gomez M, Aguayo C, Madero R, de Castro Carpeno J, Belda-Iniesta C, Barriuso J, Moreno Garcia V, Larrauri J, Lopez R, Casado E, Gonzalez-Baron M, Feliu J. KRAS mutations in primary colorectal cancer tumors and related metastases: a potential role in prediction of lung metastasis. PloS one. 2009 Dec 18;4(12):e8199.
21. Pereira AA, Rego JF, Morris V, Overman MJ, Eng C, Garrett CR, Boutin AT, Ferrarotto R, Lee M, Jiang ZQ, Hoff PM, Vauthey JN, Vilar E, Maru D, Kopetz S. Association between KRAS mutation and lung metastasis in advanced colorectal cancer. British journal of cancer. 2015 Feb 03;112(3):424-8.
22. Lipsyc M, Yaeger R. Impact of somatic mutations on patterns of metastasis in colorectal cancer. Journal of gastrointestinal oncology. 2015 Dec;6(6):645-9.
23. Russo AL, Borger DR, Szymonifka J, Ryan DP, Wo JY, Blaszkowsky LS, Kwak EL, Allen JN, Wadlow RC, Zhu AX, Murphy JE, Faris JE, Dias-Santagata D, Haigis KM, Ellisen LW, Iafrate AJ, Hong TS. Mutational analysis and clinical correlation of metastatic colorectal cancer. Cancer. 2014 May 15;120(10):1482-90.
24. Tran B, Kopetz S, Tie J, Gibbs P, Jiang ZQ, Lieu CH, Agarwal A, Maru DM, Sieber O, Desai J. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. Cancer. 2011 Oct 15;117(20):4623-32.
25. Venderbosch S, Nagtegaal ID, Maughan TS, Smith CG, Cheadle JP, Fisher D, Kaplan R, Quirke P, Seymour MT, Richman SD, Meijer GA, Ylstra B, Heideman DA, de Haan AF, Punt CJ, Koopman M. Mismatch repair status and BRAF mutation status in metastatic colorectal cancer patients: a pooled analysis of the CAIRO, CAIRO2, COIN, and FOCUS studies. Clinical cancer research : an official journal of the American Association for Cancer Research. 2014 Oct 15;20(20):5322-30.

26. Koopman M, Kortman GA, Mekenkamp L, Ligtenberg MJ, Hoogerbrugge N, Antonini NF, Punt CJ, van Krieken JH. Deficient mismatch repair system in patients with sporadic advanced colorectal cancer. British journal of cancer. 2009 Jan 27;100(2):266-73.
27. Wong SQ, Li J, Tan AY, Vedururu R, Pang JM, Do H, Ellul J, Doig K, Bell A, MacArthur GA, Fox SB, Thomas DM, Fellowes A, Parisot JP, Dobrovic A, Cohort C. Sequence artefacts in a prospective series of formalin-fixed tumours tested for mutations in hotspot regions by massively parallel sequencing. BMC Med Genomics. 2014 May 13;7:23.
28. Ludyga N, Grunwald B, Azimzadeh O, Englert S, Hofler H, Tapio S, Aubele M. Nucleic acids from long-term preserved FFPE tissues are suitable for downstream analyses. Virchows Archiv : an international journal of pathology. 2012 Feb;460(2):131-40.

Chapter 5

Tumor deposits in colorectal cancer: improving the value of modern staging - a systematic review and meta-analysis

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Abstract

Colorectal cancer (CRC) treatment is largely determined by tumor stage. Despite improvements made in the treatment of various types of metastatic disease, staging has not been refined. The role of tumor deposits (TDs) in staging remains debated. We have assessed the relation of TDs with metastatic pattern to evaluate whether TDs might add significant new information to staging.

We performed a systematic literature search that was focused on the role of TDs in CRC. Studies with neoadjuvant-treated patients were excluded. Data on stage, histological factors, and outcome were extracted. Data from four large cohorts were analyzed for the relevance of the presence of TDs, lymph node metastases (LNMs) and extramural vascular invasion (EMVI) on the pattern of metastases and outcomes.

Of the 10,106 included patients with CRC, 22% presented with TDs. TDs are invariably associated with poor outcome. Presence of TDs was associated with the presence of LNMs and EMVI. In a pairwise comparison, effects of TDs were stronger than both LNMs and EMVI. In the logistic regression model, TDs in combination with LNMs is the strongest predictor for liver (odds ratio (OR) 5.5), lung (OR 4.3) and peritoneal metastases (OR 7.0). The presence of EMVI adds information for liver and lung metastases, but not for peritoneal metastases.

We have shown that TDs are not equal to LNMs or EMVI with respect to biology and outcome. We lose valuable prognostic information by allocating TDs into nodal category N1c and only considering TDs in the absence of LNMs. Therefore, we propose that the number of TDs should be added to the number of LNMs to derive a final N stage.

Introduction

Staging of cancer is one of the cornerstones of cancer treatment. The TNM staging system is an anatomically based classification that is applied worldwide for many tumor types. Originally, this system was used to determine prognostic outcomes and to enable the international comparison of different cohorts. With increasing treatment possibilities, tumor stage has become one of the main selection criteria for (adjuvant) therapy. In colorectal cancer (CRC), stage III patients are generally treated with systemic adjuvant therapy, as are patients with high risk stage II disease.[1,2]

However, for many patients with metastatic disease, cytotoxic therapy is no longer their only treatment option and more widespread multimodality treatment with curative intent has become possible. Patients with oligometastases in liver or lung can undergo curative treatment in ever increasing numbers,[3,4] and patients with peritoneal disease can undergo cytoreduction with hyperthermic intraperitoneal chemotherapy treatment.[5] Clinical trials that will investigate treatment with adjuvant hyperthermic intraperitoneal chemotherapy in high-risk patients are currently recruiting.[6] Therefore, we need more detailed staging systems that enable a better estimation for recurrence risk at different sites to guide new treatment choices.

In recent editions of the TNM staging system, the inclusion of tumor deposits (TDs) within nodal staging has given rise to worldwide discussions.[7-12] Other important prognostic features, such as extramural vascular invasion (EMVI) are acknowledged, but not included in staging. One may wonder whether we lose useful information by ignoring the former and placing TDs with different etiologies into the nodal category, N1c, only in absence of lymph node metastases (LNMs). If TDs are equal to LNMs, both in prognostic and biological sense, this would simplify the staging systems as they can be placed in the N category without loss of information; however, if TDs add information to staging either alone or taking into account their etiology, we should apply specific sub-staging.

We assessed the prognostic impact of TDs by performing a systematic review of existing data, investigated the association of TDs with other histologic prognostic factors and determined whether TD status influenced the metastatic pattern in CRC. On the basis of the results, we propose revisions to be considered for the modern anatomical staging of CRC.

Material and methods

Strategy for search of articles and selection criteria

A comprehensive literature search for published studies was performed using Embase and Medline databases (OvidSP software) from inception to July 29th 2015, using the following keywords: "tumor deposits" or "microfoci" or "non-nodal" or "nodal independent" or "neoplastic foci" or "tumor aggregate" or "discontinuous" or "extranodal" or "staging" in combination with "Colorectal Neoplasms"[Mesh] "Cecal Neoplasms"[Mesh] or "colorectal" or "colon" or "rectum" or "rectal" and "cancer" or "carcinoma" or "tumor", limited by "Survival Analysis"[Mesh]. Additional searches were performed by manual cross-referencing.

Only original studies that were published in English with at least 100 patients were selected. In case of overlapping patient data, results of the largest study or of the study with longest follow-up were included in this meta-analysis. Studies in which histology was not reviewed for whole cohorts were excluded, since reporting on TDs without histological review is unreliable and incomplete. Studies that included patients who were treated with neoadjuvant therapy were excluded. Test and validation cohorts that have been described in the individual studies are separately analyzed.

Data extraction

For each study the number of patients in both the TD-positive and the TD-negative group were obtained. Data on tumor stage, histological factors, 5-year disease free survival (DFS), 5-year disease specific survival (DSS) and 5-year overall survival (OS) were extracted from all studies. Data were entered in SPSS (SPSS for Windows, IBM SPSS Statistics 20, 2011) and Review Manager (RevMan). Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012). Data were retrieved by two independent investigators (IN, NK).

Quality assessment and risk of bias

A scale to assess the quality of study reporting was developed on basis of the REMARK guidelines and focused on TDs.[13,14] All studies were subjected to quality assessment; studies that were only used for correlation of TDs with other factors were subjected to quality assessment in which outcome-specific items were left out. The association between the quality of reporting and the Hazard Ratio (HR) was analyzed with scatter plots and nonparametric correlation testing. Publication bias was assessed by symmetry in funnel plots.

Cohort description

Data from four cohorts was further explored to determine the association between TDs and metastatic patterns. These cohorts have been extensively described elsewhere.[9,15] In brief, the first cohort is the test cohort from the Japanese Society for Cancer of the Colon and Rectum, which included 1,716 stage I-III CRC patients who underwent curative surgery between 1994 and 1998, with an average follow-up of 93 months.[15] The validation cohort from the Japanese Society for Cancer of the Colon and Rectum included 2,242 stage I-III CRC patients who underwent curative surgery between 1999 and 2003, with an average follow-up of 68 months.[15] The UK cohort consists of 455 stage I-IV CRC patients that were included in the Medical Research Council CLASICC trial between July 1996 and July 2002, with an average follow-up of 63 months.[9] The Swedish cohort represents a consecutive case series from Falu Lasarett of 505 stage I-IV CRC patients, who underwent surgery between 1998 and 2000, with an average follow-up of 63 months.[9] Histology from all cases was reviewed with special attention for TDs, as has been described before.[9,15]

Statistical analysis

A meta-analysis was performed with all available studies on correlation in terms of risk ratios (RRs) with 95% CI. Data of univariate and multivariate analyses were entered in terms of HR with 95% CI. If no HR was reported, it was calculated from the published data,[16] but only in studies with data on minimum and maximum follow-up times. A random effects model with inverse variance weighting of studies was used. In this model each study was given a weight that was equal to the inverse of the variance of the effect estimate and served to minimize the variance of the combined effect. Forest plots are used to demonstrate consistency of the results. For effect size, Z-statistic was used (standardized mean difference). Heterogeneity was assessed using a χ^2 test for heterogeneity with a P value of <.10 to show the presence of significant heterogeneity. Furthermore, we applied I^2 statistic - percentage of variation across studies that is a result of heterogeneity rather than chance- in combination with Tau-squared - estimate of between-study variance in a random effect meta-analysis. In case of heterogeneity, subanalyses for sample size, timeframe and TNM stage were performed to identify the potential source of the heterogeneity. Logistic regression analysis was used to investigate the multivariate relationship of pathologic factors that predicted liver, lung and peritoneal metastases in the four cohorts. In the logistic regression analyses, the reference group used was the negative/ negative group, that is N0/TD negative. In the model, all first order interactions were included, adjusting for cohort, LNMs, TDs, EMVI and the combination of LNMs*TDs, LNMs*EMVI and TDs*EMVI. The model was simplified by leaving out non-statistical interactions with a p-value of >.10. Results were reported as odds ratios (ORs) with 95%CI. A p-value ≤ .05

was considered statistically significant. Hosmer and Lemeshow test for goodness of fit was used to evaluate the logistic regression models. We applied the Holm method for stepdown Bonferoni correction of multiple testing for each factor.

Results

Search results

A total of 574 studies were retrieved by the Medline database search, and 605 we found using Embase. Duplicates were excluded (n=283). A further 862 studies were excluded because they did not meet general inclusion criteria (Figure 1). We added two additional papers that fulfilled the eligibility criteria.[17,18] The remaining 36 papers concerned TDs in CRC. We excluded six studies because of insufficient patient numbers,[19-24] one gave insufficient data for analysis,[25] two studies did not perform histologic revision of all historic cases,[18,26] and seven studies had overlapping data.[12,27-32] Three studies included neoadjuvant-treated patients.[33-35]

The remaining 17 studies, which comprised 10,106 patients, were included in the meta-analysis. The main characteristics of the studies are listed in table 1.

Quality of the reporting of the included studies

Studies were subjected to quality assessment. Thirteen studies were used for meta-analysis with outcome,[15,17,36-45] of which nine studies could also be used for correlation of TDs with other factors.[15,17, 38-41, 43-45] Two additional studies had no data on outcome and were only used for correlation of TDs with other factors.[9,46] Moreover, three studies that were identified in our systematic review provided insufficient data for meta-analysis.[47-49] The mean percentage of items that were reported in studies with outcome data was 66.6% (range 39% to 84%). The mean percentage of items reported in studies with data for correlation was 71.6% (range 50% to 82%).

Frequency and impact of TDs

The average frequency of TDs for all studies was 22.0% (range 4.9% to 41.8%). Data on the impact of TDs on DFS in univariate analysis was available from five studies, which included, in total, 1,246 patients. In the presence of TDs the DFS was significantly decreased (HR 2.2, 95%CI 1.6-3.0) (Figure 2A). Considerable heterogeneity was observed among the studies (I^2=78%). With respect to the quality assessment of the studies, the percentage of items reported ranged from 50% to 84%, and this did not correlate with the magnitude of HR (Spearman r=0.56, p=0.35). Multivariate DFS analysis was available in five studies that comprised 1,536 patients and that confirmed a decreased DFS in the presence of TDs (HR 2.0, 95%CI 1.4-2.8) (Figure 2B). Substantial

heterogeneity was observed among the studies (I^2=66%). With respect to the quality assessment of the studies, the percentage of items reported ranged from 65% to 84%, and this did not correlate with the magnitude of HR (Spearman r=0.82, p=0.13).

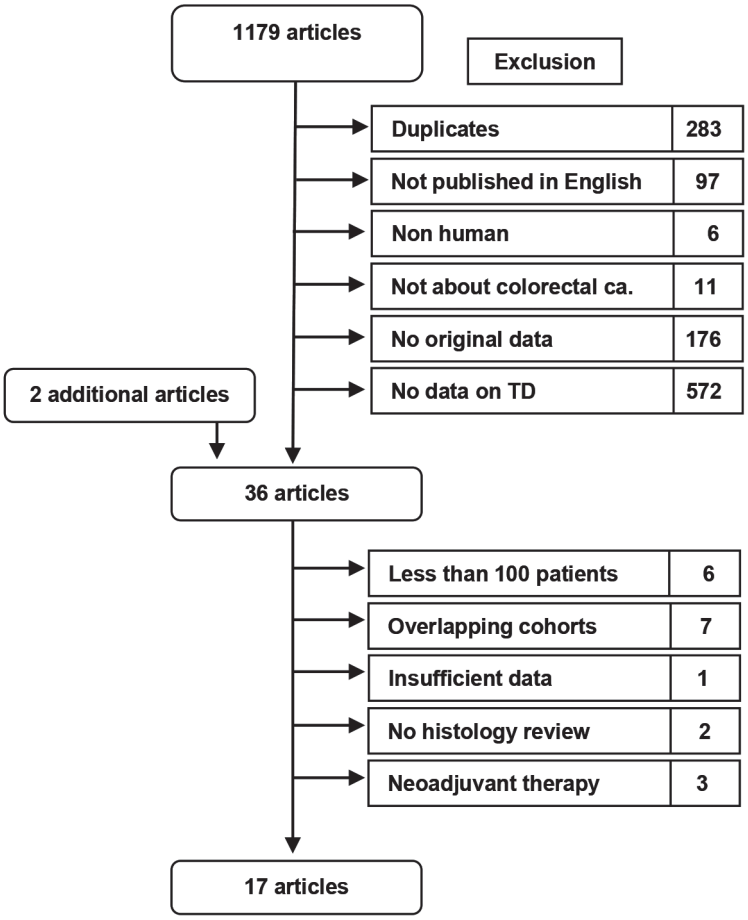


Figure 1. Flow chart of search strategy for the systematic review.
ca: cancer, TD: tumor deposit.

The effect of TDs on DSS in univariate analysis was determined in five cohorts that comprised 4,446 patients (Figure 2C) and that confirmed a decreased DSS in the presence of TDs (HR 3.3, 95%CI 2.2-4.7). Considerable heterogeneity was observed among the studies (I^2=83%). With respect to the quality assessment of the studies, the percentage of items reported ranged from 50% to 79%, and this did not correlate

Table 1. Overview of the included studies.

first author	year of publication	origin of cohort	period	stages	number of cases	% TD	location	meta-analysis correlation	meta-analysis outcome
Al Sahaf[36]	2011	Ireland	NM	III	114	28.9	colon	-	DFS UV, DSS UV+MV
Goldstein[37]	2000	USA	1973-1984	III	400	17.8	colon	-	DFS UV+MV
Harrison[49]	1994	USA	1964-1983	I-III	348	27.3	rectum	-	-
Harrison[47]	1995	USA	1965-1985	I -III	344	25.5	colon	-	-
Jin[48]	2015	USA	2001-2010	I-IV	483	28.0	colon	-	-
Lin[38]	2015	China	2003-2013	IV	146	41.8	colorectal	LNM, EMVI	DFS UV+MV, DSS UV+MV
Nagayoshi[39]	2014	Japan	1999-2006	II/III	344	10.2	colorectal	LNM, N, EMVI	DFS UV+MV, OS UV+MV
Nagtegaal[9]	2011	UK, Sweden	1996-2002	I-IV	960	34.7	colorectal	LNM, N, EMVI	-
Puppa[40]	2007	Italy	1988-1999	III-IV	228	4.9	colorectal	EMVI	DFS UV, DSS UV
Shimada[41]	2010	Japan	2000-2005	I-III	214	41.1	rectum	LNM, EMVI	DFS MV, OS MV
Song[42]	2012	China	1994-2007	III	513	29.4	colorectal	-	DSS MV
Tateishi[46]	2005	Japan	1985-1995	II-III	544	17.5	colorectal	LNM, EMVI	-
Tsutsumi[43]	2012	Japan	2005-2009	NM	263	14.4	colorectal	LNM	OS UV+MV
Ueno[17]	1997	Japan (NDMCH)	1980-1992	I-III	369	35.2	rectum	LNM	OS UV
Ueno*[12,15]	2011/2012	Japan (JSCCR)	1994-2003	I-III	3958	15.4	colorectal	LNM, N	DSS UV
von Winterfeld[44]	2014	Germany	2003-2007	I-IV	414	24.9	colorectal	LNM, N	DFS MV, DSS MV, OS MV
Yabata[45]	2014	Japan	2000-2008	I-III	464	13.1	colorectal	LNM, EMVI	OS MV
All studies					10106	22.0			

TD: tumor deposits, NM: not mentioned, LNM: lymph node metastases, N: nodal stage, EMVI: extramural vascular invasion, DFS: Disease Free Survival, DSS: Disease Specific Survival, OS: Overall Survival, UV: univariate analysis, MV: multivariate analysis
*the data of this cohort have been described in two separate papers

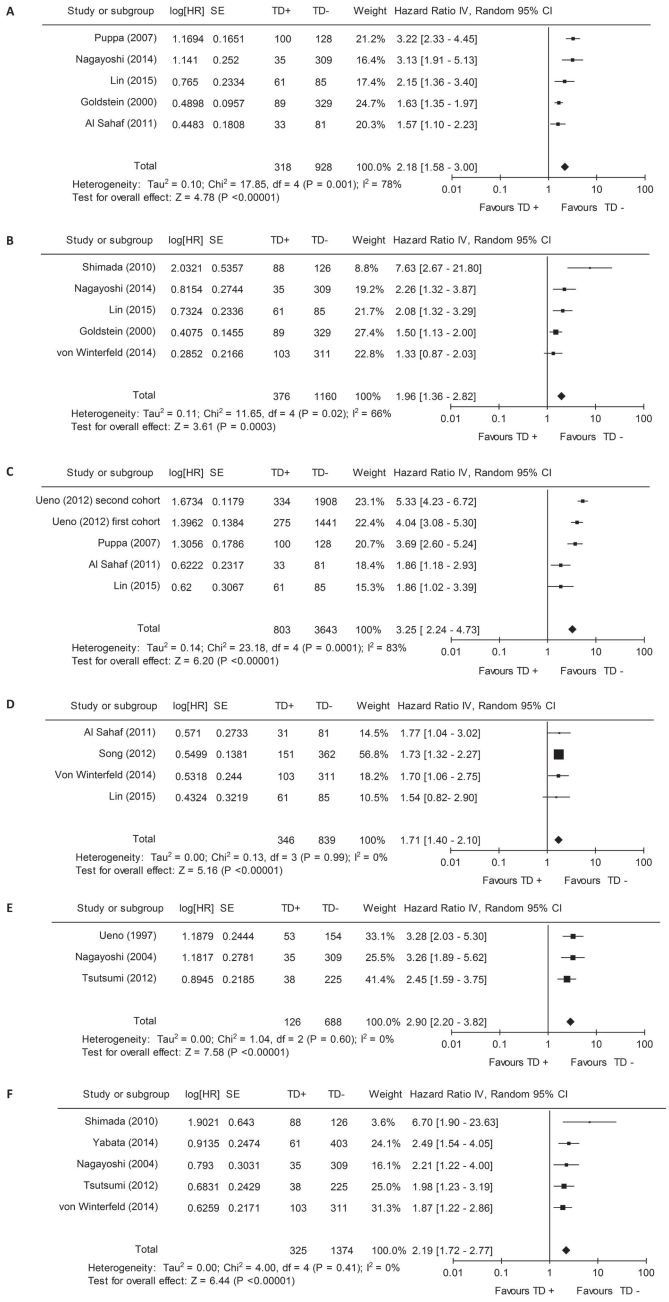


Figure 2. The impact of TD on outcome.

A-B: Disease free survival (A: univariate, B: multivariate),

C-D: Disease specific survival (C: univariate, D: multivariate),

E-F: Overall survival (E: univariate, F: multivariate).

TD-: tumor deposit negative, TD+: tumor deposit positive, HR: Hazard Ratio, SE: standard error.

with the magnitude of HR (Spearman r=-0.16, p=0.78). Multivariate DSS analysis was available in four studies that comprised 1,185 patients and confirmed a decreased DSS in the presence of TDs (HR 1.7, 95%CI 1.4-2.1) (Figure 2D). No heterogeneity was observed among the studies (I^2=0%). The percentage of items reported ranged from 50% to 79%, and this quality indicator did not correlate with the magnitude of HR (Spearman r=-0.80, p=0.33).

The impact of TDs on overall survival was available from three univariate and five multivariate cohorts, with respectively 814 and 1,699 patients (Figure 2E-F). Overall survival was decreased in the presence of TDs (univariate HR 2.9 (95%CI 2.2-3.8) and multivariate HR 2.2 (95%CI 1.7-2.8)). No heterogeneity was observed in the univariate analysis (I^2=0%) nor the multivariate analysis (I^2=0%). For the univariate studies, the percentage of items reported ranged from 56% to 84%, which did not correlate with the magnitude of HR (Spearman r=-0.50, p=1.00). For the multivariate studies, the percentage ranged between 65% and 84%, not correlating with the magnitude of HR (Spearman r=0.60, p=0.35).

Table 2. Logistic regression model for the various metastatic locations.

Factor	Liver metastases Adjusted OR (95%CI)*	Lung metastases Adjusted OR (95%CI)*	Peritoneal metastases Adjusted OR (95%CI)*
N0/TD-	1.00	1.00	1.00
N0/TD+	3.57 (2.38-5.35)	2.86 (1.71-4.78)	6.44 (3.04-13.65)
N+/TD-	2.60 (1.96-3.44)[1]	2.49 (1.81-3.44)[2]	3.21 (1.75-5.90)[3]
N+/TD+	5.54 (4.23-7.25)[1]	4.29 (3.11-5.93)[2]	6.97 (3.96-12.25)[3]
EMVI	1.38 (1.08-1.77)	2.01 (1.48-2.72)	1.25 (0.76-2.05)
Hosmer & Lemeshow Goodness of fit	p=0.476	p=0.688	p=0.498

* Data are corrected for cohort and all other listed variables. P-values using the Holm method for stepdown Bonferoni correction of multiple testing: [1]p<0.001, [2]p=0.004, [3]p=0.018. OR: odds ratio, CI: confidence interval. N0: no lymph node metastases, N+: lymph node metastases positive, TD-: tumor deposit negative, TD+: tumor deposit positive, EMVI: extramural vascular invasion

None of the analyses showed evidence of publication bias. Observed heterogeneity in DFS and DSS analyses could not be explained by differences in sample size, timeframe and TNM stage. Despite the observed heterogeneity, the direction of the effect in the forest plots is rather consistent. HR as a result of TDs is smaller in the multivariate models, as would be expected because additional variance is accounted for, however, inclusion of these additional covariates does not diminish the significance of the HR due to TDs.

Subdivisions of TD: does it matter?

The size of TDs influence prognosis; larger TDs (>12 mm in diameter) have a significantly poorer DSS (HR 2.5 and 3.2, respectively) compared to small TDs (≤3 mm).[15] Between 3 mm and 12 mm there was a nonsignificant increase in HR as a function of TD size. In another study[41] small TDs, defined as less than 2 mm, showed a very good DFS, compared to larger TDs.

The contour of TDs can be described as smooth or irregular. Two studies[11,41] of respectively 214 and 3958 patients demonstrated a trend towards poorer outcome in the irregular groups, however, no direct comparison was performed.

Increasing numbers of TDs are associated with poor outcome. In the absence of LNMs, four or more TDs are associated with a significantly shorter survival in a very small group of patients (n=17) (16.5 months versus 32.5 months, p=0.025).[48] Goldstein and Turner[38] showed that, irrespective of nodal status, the 5-year survival of patients with three or more TDs was significantly worse compared with patients with only one or two TDs (2% versus 24%, p<0.01).

Associations between TDs and histological risk factors

In 13 cohorts with a total number of 7,583 patients the relation between nodal status and the presence of TDs was studied. TDs were present in 8.7% of patients without LNMs compared with 41.6% of patients with LNMs. There were six cohorts in which the number of involved lymph nodes was studied, there was a significant increase of TDs with increasing N-stage in all studies (p=0.002, Friedman test). The risk ratio for TDs in the presence of LNMs was 4.2 (95%CI 3.2-5.6).

The relationship between TDs and EMVI (as determined by examination of hematoxylin-eosin-stained slides) was studied in nine cohorts with a total number of 2,805 patients. TDs were present in 20.9% of patients without EMVI, compared with 31.6% of patients with EMVI. RR for TDs in the presence of EMVI was 2.6 (95%CI 1.8-3.7).

Comparison of LNM, TD and EMVI

Two studies investigated the prognostic power of TDs in combination with LNMs.[41,46] Whereas absence en presence of both TDs and LNMs was associated with the best and worst outcomes, respectively, both studies suggest that the presence of only TDs is associated with a worse outcome than the presence of only LNMs.

In order to establish the value of TDs, LNMs and EMVI in modern staging, we analyzed original data from four large cohorts of studies[9,15] that were selected in this systematic review in correlation with metastatic patterns, including both synchronous and metachronous metastases. Three different metastatic patterns were distinguished: liver metastases, lung metastases, and peritoneal metastases. In the four cohorts, which had a total of 4,918 patients, there were 397 liver metastases, 268 lung metastases, and 92 peritoneal metastases. The distribution of metastases was different between the cohorts, with higher percentages of liver and peritoneal metastases in the Sweden cohort (Figure 3A).

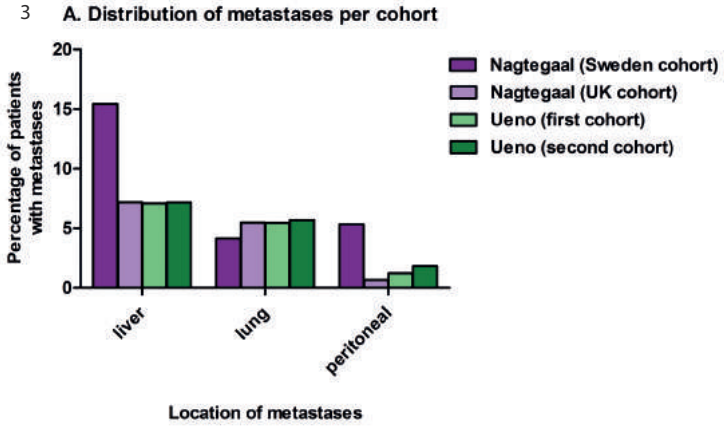


Figure 3A

For RR at different metastatic locations (Figure 3B), the effect of LNMs was similar to that of TDs; however, the combination of TDs and LNMs was associated with a significantly higher risk of liver metastases than LNMs alone. When TDs and EMVI were compared (Figure 3C), it was clear that the presence of TDs significantly increased RR of liver metastases (RR 3.6 (95%CI 2.6-5.0) versus RR 1.7 (95%CI 1.3-2.3)). RR of TDs was not different from RR of TDs and EMVI combined. For lung metastases, the combination of TDs and EMVI significantly increased RR compared with EMVI alone. When the impact of EMVI in combination with LNMs was compared, it was clear that addition of LNMs caused a higher RR for both lung and liver metastases (Figure 3D).

We subsequently evaluated the different factors by using a logistic regression model (table 2). For liver metastases, TDs, LNMs and EMVI were significant, with OR of 3.6, 2.6 and 1.4 respectively. For lung metastases, the effects of TDs, LNMs and EMVI were comparable (OR 2.9, 2.5 and 2.0, respectively). For the development of peritoneal metastases, only TDs and LNMs contributed significantly (OR 6.4 and 3.2), but not EMVI. Combination of TDs and LNMs did not increase the risk of peritoneal metastases compared with TDs alone.

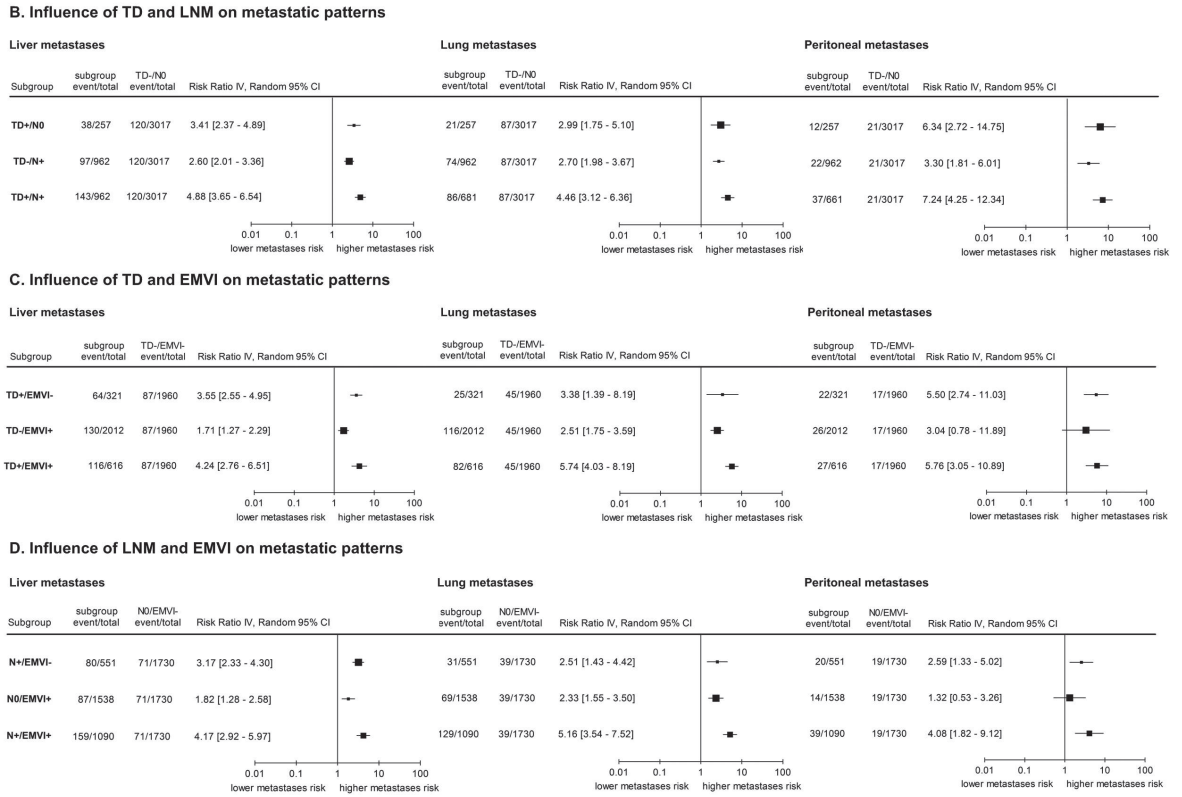


Figure 3. Metastatic patterns in relation to LNM, TD and EMVI* and combinations thereof.
A: percentage of patients with different metastatic locations in the different cohorts,
B: Influence of LNM and TD on metastatic patterns, C: Influence of EMVI and TD on metastatic patterns, D: Influence of LNM and EMVI on metastatic patterns. *According to the Japanese classification, no distinction between intramural and extramural vascular invasion is made.[58] N0: no lymph node metastases, N+: lymph node metastases positive, TD-: tumor deposit negative, TD+: tumor deposit positive, EMVI-: no extramural vascular invasion, EMVI+: extramural vascular invasion present.

Discussion

In the current systematic review, we identified 17 large-scale studies that investigated the role of TDs in CRC. In a collection of 10,106 patients with CRC, the incidence of TDs was 22%, which illustrates its potential value. The presence of TDs was invariably associated with a poorer outcome as illustrated by decreased DFS (HR 1.7-2.0), DSS (HR 1.7-3.9) and OS (HR 2.2-2.9). Some unexplained heterogeneity was present in the DFS and DSS analyses, however, the OS analyses did not show heterogeneity.

Recent editions of TNM have acknowledged the importance of TDs by incorporating it in nodal staging. In the 5th edition of TNM,[50] the size of TDs was considered important, but this was replaced by contour in the 6th edition[51] and local interpretation in the 7th edition.[52] Despite the clinical impact of these definitions, limited data are available to study both size and contour. Two studies[15,41] have confirmed that size matters by demonstrating that larger TDs are associated with worse prognosis. Data on the impact of contour is less convincing.

The correlation between TDs and other types of regional spread might be part of the explanation of the poor prognosis. TDs occur more frequently in cases with perineural invasion[21,38,40] and lymphatic invasion.[17,39-41,45,46] We summarized the most relevant correlations and demonstrated increased TDs in patients with LNMs and EMVI; however, data from multivariate studies still demonstrated an independent prognostic effect of TDs.

It is important to realize that TDs are not LNMs: the origin of TDs is diverse. By serial sectioning in a series of 30 irregular TDs[37], almost 40% showed a combined perineural, perivascular and intravascular origin. A perineural origin was present in 77% of cases and an intravascular origin in 83% of cases. A similar setup with 69 TDs[53] showed similar diversity. Presence of vessels and nerves in the majority of the TDs explains the worse prognosis of patients with TDs compared with that of patients with LNMs alone. Tumor access to more than one anatomic highway to metastatic locations creates more extensive tumor spread; therefore, we decided to evaluate the metastatic patterns that occur in the presence of TDs. The early study of Goldstein and Turner[37] suggested a significant impact of TDs in the development of intra-abdominal metastases. In their cohort, only 12% of patients without TDs developed peritoneal metastases compared with 44% of patients with TDs. In the current study, we examined original data from four different patient cohorts and the impact of TDs on the pattern of metastases. Presence of TDs and LNMs more than doubled the RR (5.3 versus 2.5) for liver metastases compared with LNMs alone. Similar trends are observed for other metastatic patterns. A first explanation would be that TDs indeed reflect EMVI, and thus explain the high risk on liver metastases;[54] however, when we compared TDs and EMVI the difference was even more pronounced. Whether there

is an unequivocal alternative biological explanation[55] remains to be investigated. From these results, it is clear that TDs do not equate to LNMs, nor recognizable EMVI, both in a prognostic and in a biological sense. This study shows that by allocating all TDs into a nodal category, pN1c, and subsequently ignoring them in the presence of LNMs, valuable prognostic information is lost. The same argument can be made for EMVI; we also lose potential information on the likely sites for recurrence.

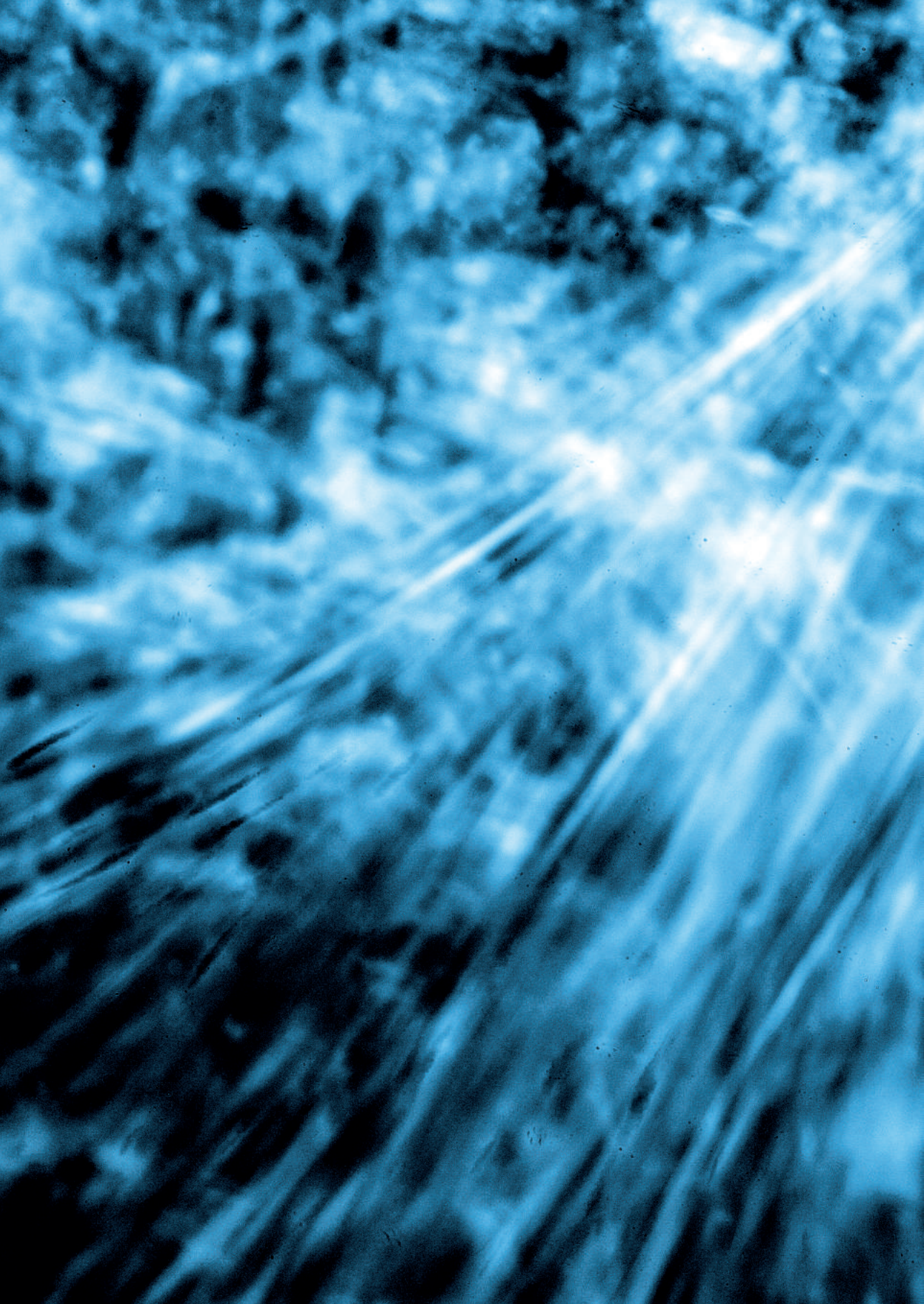
This study confirms that sufficient consistent evidence exists to now justify TD assessment in the management of CRC. However, there are a number of significant issues. The lack of definition in the current edition of TNM is not acceptable as it leads to poor inter-observer agreement.[56] True effects of the total number of TDs have not been determined, nor has this aspect been considered against the number of LNMs present. We do not know the optimal way to classify TDs after neoadjuvant treatment. Size of TDs seems to matter and further characterization is required. It is not clear how we should integrate these prognostic markers into the debate over when to use adjuvant therapy.[57] Despite all of these issues, TDs and their number should be fully included in TNM staging. Inclusion of TDs only in the absence of LNMs is not justified by the evidence. TDs and their actual number should be considered equal to the number of LNMs in making treatment decisions; therefore, the number of TDs should be added to the number of LNMs in nodal staging to derive a final N stage.

References

1. van de Velde CJ, Boelens PG, Borras JM, et al: EURECCA colorectal: multidisciplinary management: European consensus conference colon & rectum. Eur J Cancer 50:1 e1-1 e34, 2014
2. Benson AB, 3rd, Venook AP, Bekaii-Saab T, et al: Colon cancer, version 3.2014. J Natl Compr Canc Netw 12:1028-59, 2014
3. de Ridder JA, Lemmens VE, Overbeek LI, et al: Liver Resection for Metastatic Disease; A Population-Based Analysis of Trends. Dig Surg 33:104-113, 2016
4. Booth CM, Nanji S, Wei X, et al: Outcomes of Resected Colorectal Cancer Lung Metastases in Routine Clinical Practice: A Population-Based Study. Ann Surg Oncol, 2015
5. Elias D, Lefevre JH, Chevalier J, et al: Complete cytoreductive surgery plus intraperitoneal chemohyperthermia with oxaliplatin for peritoneal carcinomatosis of colorectal origin. J Clin Oncol 27:681-5, 2009
6. Klaver CE, Musters GD, Bemelman WA, et al: Adjuvant hyperthermic intraperitoneal chemotherapy (HIPEC) in patients with colon cancer at high risk of peritoneal carcinomatosis; the COLOPEC randomized multicentre trial. BMC Cancer 15:428, 2015
7. Nagtegaal ID, Quirke P: Revised staging: is it really better, or do we not know? J Clin Oncol 28:e397-8; author reply e399-400, 2010
8. Nagtegaal ID, Quirke P, Schmoll HJ: Has the new TNM classification for colorectal cancer improved care? Nat Rev Clin Oncol 9:119-23, 2012
9. Nagtegaal ID, Tot T, Jayne DG, et al: Lymph nodes, tumor deposits, and TNM: are we getting better? J Clin Oncol 29:2487-92, 2011
10. Quirke P, Williams GT, Ectors N, et al: The future of the TNM staging system in colorectal cancer: time for a debate? Lancet Oncol 8:651-7, 2007
11. Ueno H, Mochizuki H, Shirouzu K, et al: Multicenter study for optimal categorization of extramural tumor deposits for colorectal cancer staging. Annals of Surgery 255:739-746, 2012
12. Ueno H, Mochizuki H, Akagi Y, et al: Optimal colorectal cancer staging criteria in TNM classification. Journal of Clinical Oncology 30:1519-26, 2012
13. Knijn N, Simmer F, Nagtegaal ID: Recommendations for reporting histopathology studies: a proposal. Virchows Arch, 2015
14. McShane LM, Altman DG, Sauerbrei W, et al: Reporting recommendations for tumor marker prognostic studies. J Clin Oncol 23:9067-72, 2005
15. Ueno H, Mochizuki H, Shirouzu K, et al: Actual status of distribution and prognostic impact of extramural discontinuous cancer spread in colorectal cancer. Journal of Clinical Oncology 29:2550-2556, 2011
16. Parmar MK, Torri V, Stewart L: Extracting summary statistics to perform meta-analyses of the published literature for survival endpoints. Stat Med 17:2815-34, 1998
17. Ueno H, Mochizuki H: Clinical significance of extrabowel skipped cancer infiltration in rectal cancer. Surg Today 27:617-22, 1997
18. Belt EJ, van Stijn MF, Bril H, et al: Lymph node negative colorectal cancers with isolated tumor deposits should be classified and treated as stage III. Ann Surg Oncol 17:3203-11, 2010
19. Ishikawa K, Hashiguchi Y, Mochizuki H, et al: Extranodal cancer deposit at the primary tumor site and the number of pulmonary lesions are useful prognostic factors after surgery for colorectal lung metastases. Diseases of the Colon and Rectum 46:629-636, 2003
20. Kiss L, Kiss R, Porr PJ, et al: Pathological evidence in support of total mesorectal excision in the management of rectal cancer. Chirurgia (Bucuresti) 106:347-52, 2011
21. Prabhudesai A, Arif S, Finlayson CJ, et al: Impact of Microscopic Extranodal Tumor Deposits on the Outcome of Patients with Rectal Cancer. Diseases of the Colon and Rectum 46:1531-1537, 2003
22. Ratto C, Ricci R, Rossi C, et al: Mesorectal microfoci adversely affect the prognosis of patients with rectal cancer. Diseases of the Colon and Rectum 45:733-742, 2002

23. Tocchi A, Mazzoni G, Lepre L, et al: Total mesorectal excision and low rectal anastomosis for the treatment of rectal cancer and prevention of pelvic recurrences. Archives of Surgery 136:216-220, 2001
24. Ueno H, Mochizuki H, Fujimoto H, et al: Autonomic nerve plexus involvement and prognosis in patients with rectal cancer. British Journal of Surgery 87:92-96, 2000
25. Prall F, Schmitt O, Schiffmann L: Tumor regression in rectal cancer after intensified neoadjuvant chemoradiation: a morphometric and clinicopathological study. World Journal of Surgical Oncology 13:155, 2015
26. Lo DS, Pollett A, Siu LL, et al: Prognostic significance of mesenteric tumor nodules in patients with stage III colorectal cancer. Cancer 112:50-54, 2008
27. Lin Q, Ye Q, Zhu D, et al: Determinants of long-term outcome in patients undergoing simultaneous resection of synchronous colorectal liver metastases. PLoS ONE 9, 2014
28. Puppa G, Ueno H, Kayahara M, et al: Tumor deposits are encountered in advanced colorectal cancer and other adenocarcinomas: An expanded classification with implications for colorectal cancer staging system including a unifying concept of in-transit metastases. Modern Pathology 22:410-415, 2009
29. Tong LL, Gao P, Wang ZN, et al: Is the seventh edition of the UICC/AJCC TNM staging system reasonable for patients with tumor deposits in colorectal cancer? Annals of Surgery 255:208-213, 2012
30. Ueno H, Hashiguchi Y, Shimazaki H, et al: Peritumoral deposits as an adverse prognostic indicator of colorectal cancer. American Journal of Surgery 207:70-77, 2014
31. Ueno H, Mochizuki H, Hashiguchi Y, et al: Extramural cancer deposits without nodal structure in colorectal cancer: Optimal categorization for prognostic staging. American Journal of Clinical Pathology 127:287-294, 2007
32. Ueno H, Mochizuki H, Tamakuma S: Prognostic significance of extranodal microscopic foci discontinuous with primary lesion in rectal cancer. Diseases of the Colon and Rectum 41:55-61, 1998
33. Gopal P, Lu P, Ayers GD, et al: Tumor deposits in rectal adenocarcinoma after neoadjuvant chemoradiation are associated with poor prognosis. Modern Pathology 27:1281-1287, 2014
34. Song JS, Chang HJ, Kim DY, et al: Is the N1c category of the new american joint committee on cancer staging system applicable to patients with rectal cancer who receive preoperative chemoradiotherapy? Cancer 117:3917-3924, 2011
35. Swellengrebel HA, Bosch SL, Cats A, et al: Tumour regression grading after chemoradiotherapy for locally advanced rectal cancer: a near pathologic complete response does not translate into good clinical outcome. Radiotherapy & Oncology 112:44-51, 2014
36. Al Sahaf O, Myers E, Jawad M, et al: The prognostic significance of extramural deposits and extracapsular lymph node invasion in colon cancer. Diseases of the Colon & Rectum 54:982-8, 2011
37. Goldstein NS, Turner JR: Pericolonic tumor deposits in patients with T3N+MO colon adenocarcinomas: markers of reduced disease free survival and intra-abdominal metastases and their implications for TNM classification. Cancer 88:2228-38, 2000
38. Lin Q, Wei Y, Ren L, et al: Tumor deposit is a poor prognostic indicator in patients who underwent simultaneous resection for synchronous colorectal liver metastases. OncoTargets and Therapy 8:233-240, 2015
39. Nagayoshi K, Ueki T, Nishioka Y, et al: Tumor deposit is a poor prognostic indicator for patients who have stage II and III colorectal cancer with fewer than 4 lymph node metastases but not for those with 4 or more. Diseases of the Colon and Rectum 57:467-474, 2014
40. Puppa G, Maisonneuve P, Sonzogni A, et al: Pathological assessment of pericolonic tumor deposits in advanced colonic carcinoma: relevance to prognosis and tumor staging. Modern Pathology 20:843-55, 2007
41. Shimada Y, Takii Y: Clinical impact of mesorectal extranodal cancer tissue in rectal cancer: Detailed pathological assessment using whole-mount sections. Diseases of the Colon and Rectum 53:771-778, 2010
42. Song YX, Gao P, Wang ZN, et al: Can the tumor deposits be counted as metastatic lymph nodes in the UICC TNM staging system for colorectal cancer? PLoS ONE 7, 2012

43. Tsutsumi S, Watanabe R, Tabe Y, et al: Extranodal metastasis predicts poor survival in advanced colorectal cancer. Hepato-Gastroenterology 59:372-374, 2012
44. Von Winterfeld M, Hoffmeister M, Ingold-Heppner B, et al: Frequency of therapy-relevant staging shifts in colorectal cancer through the introduction of pN1c in the 7th TNM edition. European Journal of Cancer 50:2958-2965, 2014
45. Yabata E, Udagawa M, Okamoto H: Effect of tumor deposits on overall survival in colorectal cancer patients with regional lymph node metastases. Journal of Rural Medicine 9:20-6, 2014
46. Tateishi S, Arima S, Futami K, et al: A clinicopathological investigation of "tumor nodules" in colorectal cancer. Surg Today 35:377-84, 2005
47. Harrison JC, Dean PJ, El-Zeky F, et al: Impact of the Crohn's-like lymphoid reaction on staging of right-sided colon cancer: Results of multivariate analysis. Human Pathology 26:31-38, 1995
48. Jin M, Roth R, Rock JB, et al: The impact of tumor deposits on colonic adenocarcinoma AJCC TNM staging and outcome. American Journal of Surgical Pathology 39:109-15, 2015
49. Harrison JC, Dean PJ, el Zeky F, et al: From Dukes through Jass: pathological prognostic indicators in rectal cancer. Hum.Pathol 25:498-505, 1994
50. Sobin LH, Wittekind C: UICC TNM classification of malignant tumours (ed 5th). New York, John Wiley & sons,Inc., 1997
51. Sobin LH, Wittekind C: TNM classification of malignant tumours (ed 6th edition). New York, Wiley-Liss, 2002
52. Sobin LH, Gospodarowicz M, Wittekind C: TNM classification of malignant tumours (ed Seventh edition). Circhester, John Wiley & Sons, 2009
53. Wunsch K, Muller J, Jahnig H, et al: Shape is not associated with the origin of pericolonic tumor deposits. Am J Clin Pathol 133:388-94, 2010
54. Talbot IC, Ritchie S, Leighton MH, et al: The clinical significance of invasion of veins by rectal cancer. Br.J.Surg. 67:439-442, 1980
55. Fan XJ, Wan XB, Yang ZL, et al: Snail promotes lymph node metastasis and Twist enhances tumor deposit formation through epithelial-mesenchymal transition in colorectal cancer. Hum Pathol 44:173-80, 2013
56. Rock JB, Washington MK, Adsay NV, et al: Debating deposits: an interobserver variability study of lymph nodes and pericolonic tumor deposits in colonic adenocarcinoma. Arch Pathol Lab Med 138:636-42, 2014
57. Pahlman LA, Hohenberger WM, Matzel K, et al: Should the Benefit of Adjuvant Chemotherapy in Colon Cancer Be Re-Evaluated? J Clin Oncol 34:1297-9, 2016
58. JSCCR: Japanese classification of colorectal carcinoma (ed second English edition). Tokyo, Kanehara & co, Ltd, 2009



Chapter 6

Limited effect of lymph node status on the metastatic pattern in colorectal cancer

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Abstract

Regional lymph node metastases in colorectal cancer (CRC) decrease outcome. Whether nodal metastases function as a biomarker, i.e. as a sign of advanced disease, or are in fact involved in the metastatic process is unclear. We evaluated metastatic patterns of CRC according to the lymph node status of the primary tumor.

A retrospective review of 1393 patients with metastatic CRC who underwent autopsy in the Netherlands was performed. Metastatic patterns of regional lymph node positive and negative CRC were compared and validated by population-based data from the Eindhoven Cancer Registry (ECR).

Patients with regional lymph node positive CRC more often developed peritoneal metastases (28% vs. 21%, p=0.003) and distant lymph node metastases (25% vs. 15%, p<0.001). Incidences of liver and lung metastases were comparable. Data from the ECR confirmed our findings regarding peritoneal (22.4% vs. 17.0%, p=0.003) and distant lymph node metastases (15.8% vs. 9.7%, p<0.001).

Regional lymph node positive CRC show a slightly different dissemination pattern, with higher rates of peritoneal and distant lymph nodes metastases. Comparable incidences of liver and lung metastases support the hypothesis that dissemination to distant organs occurs independently of lymphatic spread.

Introduction

Despite intensive follow-up and increasing therapeutic options for colorectal cancer (CRC), metastatic disease remains the leading factor in CRC mortality. CRC most frequently metastasizes to the liver, lung and peritoneum, but other metastatic sites such as bone, spleen, brain and distant lymph nodes have been described.[1-3]

According to the mechanistical view of metastatic spread, tumor cells can disseminate to distant organs through two pathways: the vascular and the lymphatic pathway. The vascular hypothesis suggest that blood vessels transport tumor cells directly to distant organs. In the lymphatic pathway tumor cells may disseminate from regional lymph nodes to distant lymph nodes, reach the systemic circulation and subsequently form organ metastases.[4] The distinction between these pathways and their role in dissemination remains matter of debate.

Post-mortem studies offer a possibility to register both the extent and location of metastatic disease. Findings during autopsy may be considered the ultimate endpoint of disease. Autopsy studies are therefore usefull for getting insight in the relevance of lymphatic spread in the dissemination of cancer. Most autopsy studies, have focused on metastatic patterns in one or more types of cancer, but have failed to address differentiating aspects such as lymph node involvement. A large autopsy study by Budczies et al. across major cancer types, showed higher rates of metastases in distant lymph nodes, peritoneal cavity, pleura, pericardial and adrenal glands in lymph node positive tumors.[5] Although this study has given insight into metastatic patterns, this was done by grouping various cancers together.

To gain insight in the relevance of lymphatic spread in the dissemination of CRC, we evaluated patterns of metastases according to the lymph node status of the primary tumor in 1393 autopsies. To confirm the clinical relevance, we analyzed population-based data from the Eindhoven Cancer Registry.

Material and methods

Study design

An autopsy cohort was selected to compare patterns of metastases according to the regional lymph node status of the primary tumor. Findings at autopsy are the ultimate endpoint of disease, making autopsy reports suitable for analyzing the extend of disease. Autopsy data are derived from a restricted patient population, therefore a prospectively collected cohort of the Eindhoven Cancer Registry (ECR) was chosen for validation. First, differences in metastatic pattern according to the lymph node status of the primary tumor were analysed. Due to the close relationship between lymphatic spread,

regional and distant lymph node metastases, separate analyses were performed in tumors with and without distant lymph node metastases in the autopsy cohort.

Autopsy cohort

A total of 1679 patients with metastatic colorectal cancer was identified in an autopsy study by Hugen et al.[6] Data from this study was used for the present analyses. Patients were selected from a retrospective review of pathological and autopsy records from the nationwide network and registry of histo- and cytopathology in the Netherlands (Pathologisch-Anatomisch Landelijk Geautomatiseerd Archief; PALGA)[7]

Patients who were diagnosed with metastatic CRC and autopsied between 1991 and 2010 were selected. In the Netherlands post-mortem examination is performed at the request of the family or treating doctor and is carried out by a pathologist. All autopsies included in this study were performed in order to obtain information on the medical status of the deceased or to determine the exact cause of death. No forensic autopsies were included. Tumor histology was assessed by different pathologists. Local staging of the primary tumor was reconstructed according to the TNM classification (5th edition).[8] Allocation to the lymph node category was based on pathological examination of the original resection specimen or on the autopsy specimen. For regional lymph nodes metastasis only positive lymph nodes along the colon or rectum, plus the nodes along the major arteries that supply blood to the colon or rectum were considered. Metastasis in all other nodes were considered distant lymph nodes metastases. Patients of whom the primary N-stage could not be retrieved were excluded (n=286). Colon tumors were classified as proximal if they were found in cecum, ascending colon or transverse colon, and classified as distal if they were found in the descending or sigmoid colon. Data on gender and age were available for all cases, but further clinical information (e.g. treatment or disease course) was lacking in this database and could not be retrieved. Metastatic disease was determined during pathological assessments of resected or biopsied specimens during follow-up or during autopsy. All metastases found at autopsy were histologically confirmed. Metastases that were detected more than six months after surgery of the primary tumor were considered metachronous.[9]

Clinical cohort

Data were retrieved from the Eindhoven Cancer Registry (ECR) which collects data of all patients with newly diagnosed cancer in the southeastern part of the Netherlands.[10] All patients who were diagnosed with CRC between 2003 and 2008 were included if they had synchronous metastases or developed metastases during follow-up untill 2010-2011 (n=3092). End of follow-up was defined as the date of death or end of data collection in 2010-2011.

Patients who underwent an autopsy were excluded to prevent overlap with the initial cohort (n=37). Moreover, patients with missing N-stage (n=673) were excluded. Tumor staging, classification of primary tumor location and onset of metastatic disease were performed as described for the autopsy cohort. Allocation to the lymph node category was based on pathological examination of the original resection specimen. Anatomical sites of metastases were registered according to the International Classification of Diseases for Oncology (ICD-O).[11] Patterns of metastatic disease were determined based on the first site of metastasis.

Statistical analysis

The χ^2 test was used to compare baseline characteristics between regional lymph node positive and negative CRC. Logistic regression analysis was used to analyze patient and tumor characteristics associated with location specific metastases. This analysis was performed in the clinical cohort, because of potential bias in the autopsy cohort. Odds ratio's (ORs) were provided with their 95% confidence interval (CI). In multivariate (MV) analyses adjustments were made for age, gender, primary T-stage, primary N-stage, differentiation grade of primary tumor, localization of primary tumor, primary tumor histology and onset of metastases. Statistical analyses were performed using SAS/STAT1 statistical software (SAS system 9.3, SAS Institute, Cary, North Carolina, USA) and the statistical software package SPSS 20.0 (SPSS Inc, Chicago, Illinois, USA). All tests of significance were two sided and differences at P-values of ≤0.05 were considered to be significant.

Results

In the autopsy cohort, there were 1393 patients with metastatic disease; 879 patients (63%) with regional lymph node metastases (N+) and 514 patients (37%) without regional lymph node involvement (N-). The distribution of patient and tumor characteristics according to regional lymph node status is presented in Table 1. N+ patients more often had a higher T-stage (T3/T4: 90.8% vs. 85.4%, p<0.001), a tumor located in the proximal part of the colon (38.3% vs. 31.5%, p=0.01), mucinous or signet ring cell histology (20.9% vs. 14.2%, p=0.002), multiple metastases (56.5% vs. 43.2%, p<0.001) and synchronous onset of metastases (61.2% vs. 36.2%, p<0.001).

Distribution of metastases

The liver was the most frequent site of metastasis irrespective of regional lymph node status (68% in N+ and 67% in N-, p=0.53). Lung metastases occurred in 33% of N- patients and in 35% of N+ patients (p=0.42; Figure 1A). There was a higher rate

of peritoneal metastases in N+ patients (28% vs. 21%, p=0.003) and distant lymph node metastases were more often found in N+ patients (25% vs. 15%, p<0.001). Other significant differences were found for metastases in omentum, spleen and pancreas (N+ vs. N-; 9.2 vs. 3.3%, p<0.001, 2.8 vs. 1.0%, p=0.02, and 2.6 vs. 1.0%, p=0.04, respectively).

Table 1. Distribution of tumor and patient characteristics according to regional lymph node status of the primary tumor in the autopsy cohort.

Features	**N+**		**N-**		***P*-value**
	879	**(%)**	**514**	**(%)**	
Gender					0.375
Male	514	58.5	313	60.9	
Female	365	41.5	201	39.1	
Age at diagnosis					0.681
<60	188	21.4	96	18.7	
60-74	408	46.4	247	48.1	
≥75	283	32.2	171	33.3	
Location of primary					0.032
Proximal colon	337	38.3	162	31.5	
Distal colon	271	30.8	162	31.5	
Rectum	203	23.1	135	26.3	
Colon, not specified	68	7.7	55	10.7	
T Stage					<0.001
T1	0	0	7	1.4	
T2	37	4.2	53	10.3	
T3	605	68.8	338	65.8	
T4	193	22.0	101	19.6	
Not specified	43	4.9	15	2.9	
Histology					0.002
Non-mucinous adenocarcinoma	695	79.1	441	85.8	
Mucinous adenocarcinoma	156	17.7	68	13.2	
Signet ring cell	28	3.2	5	1.0	
Onset of metastases					<0.001
Synchronous	538	61.2	186	36.2	
Metachronous	341	38.8	328	63.8	
Number of distant metastases					<0.001
1	382	43.5	292	56.8	
>1	497	56.5	222	43.2	

N+: primary tumor with regional lymph node metastases;
N-: primary tumor without regional lymph node metastases.

When patients were subdivided according to the location of the primary tumor, a higher percentage of lung metastases in N+ rectal cancer as compared to N+ colon cancer (43.8% vs. 32.1%, p=0.001) and higher percentage of peritoneal metastases in N+ colon cancer as compared to N+ rectal cancer (30.8% vs. 19.2%, p=0.002) was found. This could be related to the higher overall percentage of lung metastases in rectal cancer compared with colon cancer (42.6% vs. 31.3%, p<0.001) and of peritoneal metastases in colon cancer compared with rectal cancer (28.1% vs. 17.2%, p<0.001). N+ colon cancers more often had peritoneal metastases and distant lymph node metastases compared with N- colon cancers (24.0% vs. 14.0%, p<0.001, and 30.8% vs. 23.2%, p=0.009, respectively). In rectal cancer only distant lymph node metastases were more often seen in N+ than in N- patients (28.1% vs. 18.5%, p=0.05).

Validation of findings

A total of 2382 patients with metastatic colorectal cancer from the Eindhoven Cancer Registry (ECR) was included, of which 1711 patients (71.8%) had regional lymph node metastases and 671 patients (28.2%) did not. Median follow-up was 5.0 years (range 1.2–8.8 years). The distribution of patient and tumor characteristics according to lymph node status is presented in Table 2.

N+ patients more often developed peritoneal metastases and distant lymph node metastases (22.4% vs. 17.0%, p=0.003, and 15.8% vs. 9.7%, p<0.001). Moreover, there was a higher percentage of liver metastases and a lower percentage of lung metastases in N+ patients compared with N- patients (71.6% vs. 66.3%, p=0.01 and 23.7% vs. 27.7%, p=0.04, respectively).

Logistic regression analyses identified several clinicopathological factors that were associated with location specific metastases (Table 3). Rectal tumors were associated with a higher risk of developing liver and lung metastases (OR for liver: 1.33 (1.05-1.67), p<0.05, OR for lung: 2.25 (1.74-2.89), p<0.001), and with a lower risk of developing peritoneal carcinomatosis (OR: 0.24 (0.18-0.32), p<0.001). T4 tumors less often led to liver metastases (OR: 0.48 (0.38-0.61), p<0.001) and were associated with a higher risk of developing peritoneal carcinomatosis (OR: 2.18 (1.72-2.77), p<0.001). Lymph node positive tumors were associated with an increased risk of developing distant lymph node metastases, especially N2 tumors (OR: 3.03 (2.14-4.29), p<0.001). N2 tumors were also associated with peritoneal carcinomatosis (OR: 1.40 (1.05-1.87), p<0.05). Mucinous tumors less often led to liver metastases (OR: 0.46 (0.34-0.62), p<0.001) and were associated with a higher risk of developing peritoneal carcinomatosis (OR: 2.53 (1.84-3.47), p<0.001).

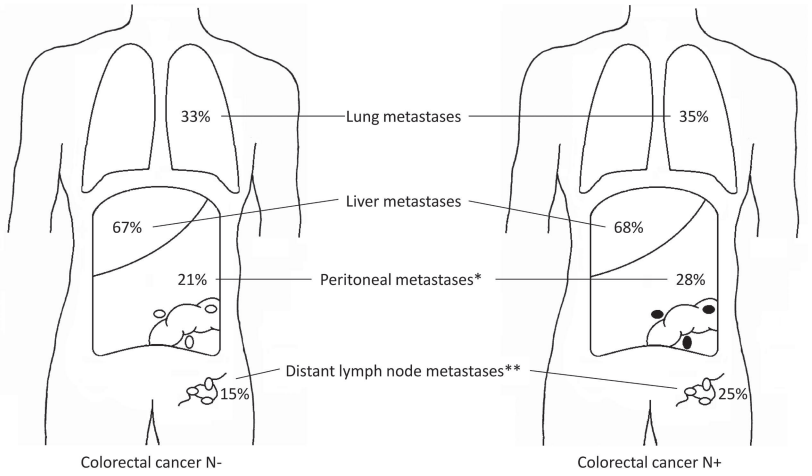

Figure 1A. Distribution of CRC metastases according to regional lymph node status in the autopsy cohort. Left figure shows the distribution of metastases for regional lymph node negative primary tumors, right figure shows the distribution of metastases for regional lymph node positive primary tumors. * p=0.003, ** p<0.001

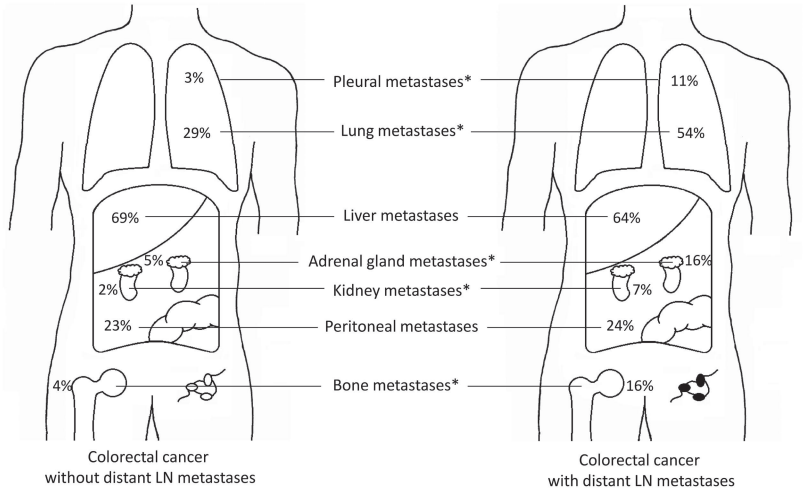

Figure 1B. Distribution of CRC metastases according to distant lymph node positivity in the autopsy cohort. Left figure shows the distribution of metastases for primary tumors without distant lymph node metastases, right figure shows the distribution of metastases for primary tumors with distant lymph node metastases. * p<0.001

Table 2. Distribution of tumor and patient characteristics according to regional lymph node status of the primary tumor in the clinical cohort.

Features	N+		N-		*P*-value
	1711	(%)	671	(%)	
Gender					0.583
Male	952	55.6	365	54.4	
Female	759	44.4	306	45.6	
Age at diagnosis					0.008
<60	448	26.2	135	20.1	
60-74	814	47.6	342	51.0	
≥75	449	26.2	194	28.9	
Location of primary					0.014
Proximal colon	609	35.6	192	28.6	
Distal colon	504	29.5	216	32.2	
Rectum	572	33.4	252	37.6	
Colon, unknown	26	1.5	11	1.6	
T Stage					<0.001
T1	7	0.4	12	1.8	
T2	83	4.8	89	13.3	
T3	1101	64.4	411	61.2	
T4	397	23.2	106	15.8	
Unknown	123	7.2	53	7.9	
Histology					0.230
Non-mucinous adenocarcinoma	1562	91.3	621	92.5	
Mucinous adenocarcinoma	121	7.1	45	6.7	
Signet ring cell	28	1.6	5	0.8	
Onset of metastases					<0.001
Synchronous	1155	67.5	318	47.4	
Metachronous	556	32.5	353	52.6	
Number of distant metastases					<0.001
1	1067	62.4	460	68.6	
>1	644	37.6	211	31.4	

N-: primary tumor without regional lymph node metastases;
N+: primary tumor with regional lymph node metastases

Distant lymph node metastases

To provide further insight into the relevance of regional lymph node metastases for distant lymph node metastases, data was analyzed from 1024 cases with at least ten lymph nodes retrieved with the primary tumor. We found an increasing rate of distant lymph node metastases according to the number of positive lymph nodes detected in the primary tumor (Figure 2). Data from the ECR showed an increase from 9.1% in patients without positive lymph nodes to 27.1% in patients with more than twelve positive lymph nodes detected in the primary tumor ($p<0.001$). Data from the autopsy study showed an increase from 8.2% to 48.4% ($p<0.001$).

Compared with patients without distant lymph node metastases, patients with distant lymph node metastases more often had metastases in lung (57% vs. 29%, $p<0.001$), pleura (11% vs. 3%, $p<0.001$), bone (16% vs. 4%, $p<0.001$), adrenal gland (16% vs. 5%, $p<0.001$) and kidney (7% vs. 2%, $p<0.001$) (Figure 1B).

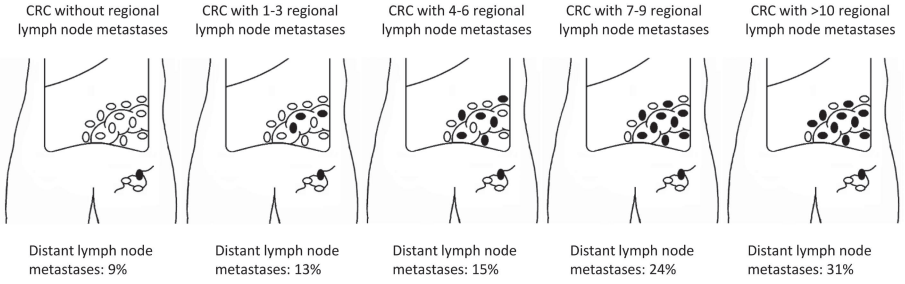

Figure 2. Percentage of patients with distant lymph node metastases according to the number of positive lymph nodes in primary tumor. Selection of patients with more than ten lymph nodes examined (N=1024 pts; autopsy cohort=258 pts; clinical cohort=766 pts), $p<0.001$

Table 3: Risk of developing distant metastases in the clinical cohort

Clinicopathological factors	Risk of developing liver metastases	Risk of developing lung metastases	Risk of developing peritoneal carcinomatosis	Risk of developing distant lymph node metastases
	MV analyses OR (95% CI)	MV analyses OR (95% CI)	MV analyses OR (95% CI)	MV analyses OR (95% CI)
Age				
<59	1.00	1.00	1.00	1.00
60-74	0.99 (0.78-1.25)	1.26 (0.98-1.61)	0.85 (0.65-1.11)	0.87 (0.65-1.16)
≥75	0.96 (0.74-1.25)	1.13 (0.85-1.50)	0.98 (0.73-1.31)	**0.67 (0.48-0.94)***
Gender				
Male	1.00	1.00	1.00	1.00
Female	**0.60 (0.50-0.72)*****	1.02 (0.83-1.24)	1.19 (0.96-1.47)	1.15 (0.90-1.46)
Location of tumor				
Proximal colon	1.00	1.00	1.00	1.00
Distal colon	**1.64 (1.30-2.08)*****	**1.39 (1.06-1.81)***	**0.60 (0.47-0.76)*****	0.88 (0.65-1.20)
Rectum	**1.33 (1.05-1.67)***	**2.25 (1.74-2.89)*****	**0.24 (0.18-0.32)*****	0.78 (0.58-1.06)
not specified	0.76 (0.37-1.54)	1.43 (0.63-3.23)	1.20 (0.59-2.46)	0.88 (0.35-2.23)
T-stage				
T1-2	1.23 (0.86-1.76)	1.00 (0.71-1.42)	**0.42 (0.23-0.77)****	1.17 (0.75-1.84)
T3	1.00	1.00	1.00	1.00
T4	**0.48 (0.38-0.61)*****	1.10 (0.84-1.43)	**2.18 (1.72-2.77)*****	1.28 (0.94-1.74)
not specified	1.14 (0.74-1.75)	1.32 (0.87-2.01)	1.26 (0.82-1.95)	**3.76 (2.38-5.95)*****
N-stage				
N0	1.00	1.00	1.00	1.00
N1	1.13 (0.90-1.41)	1.25 (0.99-1.59)	1.14 (0.87-1.49)	**1.97 (1.43-2.72)*****
N2	1.23 (0.95-1.58)	0.92 (0.70-1.22)	**1.40 (1.05-1.87)***	**3.03 (2.14-4.29)*****
Histology				
Non-mucinous adenoca	1.00	1.00	1.00	1.00
Mucinous adenoca	**0.46 (0.34-0.62)*****	0.74 (0.52-1.05)	**2.53 (1.84-3.47)*****	1.20 (0.82-1.77)
Onset of metastases				
Synchronous	1.00	1.00	1.00	1.00
Metachronous	**0.38 (0.31-0.47)*****	**4.10 (3.31-5.09)*****	1.09 (0.86-1.37)	**4.35 (3.31-5.71)*****

In multivariate analyses adjustments were made for age, gender, location of the primary tumor, primary tumor stage, primary lymph node stage, tumor histology and onset of metastases. *p<0.05, **p<0.01, ***p<0.001. Abbreviations: MV: multivariate; OR: Odds Ratio; adenoca: adenocarcinoma.

Discussion

This is the first large study comparing metastatic patterns according to lymph node status of CRC. The most common site of distant metastasis was liver followed by lung, peritoneum and distant lymph nodes, with percentages comparable to literature.[2, 12] Peritoneal and distant lymph node metastases occurred more often in regional lymph node positive CRC, while liver and lung metastases occurred in a similar percentage. Our multivariate analysis shows that next to established risk factors for peritoneal

carcinomatosis, like T-stage, proximal location and mucinous carcinoma, regional lymph node metastases are an important risk factor. This is in line with other studies[13-16] making it likely that lymph node metastases are involved in the etiology of peritoneal carcinomatosis. The omentum is a preferential site of peritoneal metastases and the lymphoid milky spots in the omentum are a homing site for metastatic cancer cells.[17] Tumor cells in the omentum can reach the peritoneal cavity by direct growth. The milky spots and the anti-inflammatory function of the omentum suggest a relation between the peritoneum and the lymphatic system. Moreover, chylous ascites can occur after obstruction or resection of extra-peritoneal located lymphatic vessels, providing more evidence for direct communications between lymphatic vessels and the peritoneal cavity.[18]

The finding that regional lymph node positive CRC spread more often to distant lymph nodes is in line with others[5] and in support of the first part of the lymphatic hypothesis. However, since no difference was observed in liver metastases, the vascular hypothesis seems more important for liver metastases.[4] Based on the lymphatic hypothesis we expected that lymphatic drainage of the thoracic duct into the venous system would lead to a higher incidence of lung metastases in regional lymph node positive CRC. However, we found comparable incidences of lung metastases in the whole group of patients with regional lymph node positive CRC (n = 1590). In a subgroup of patients with *distant* lymph node metastases analysed in the autopsy cohort (n = 297), we did find increased lung and pleural metastases, suggesting that for this small subgroup the lymphatic pathway is important for the metastatic pattern.

Our unique setup, where we validated findings from autopsy studies in a registry based cohort with prospectively collected data, illustrated only a limited influence of lymph node metastases on metastatic patterns in CRC. Autopsy studies contain selected populations, in which patients are included who have died postoperatively, had an unexpected clinical course, or died of other causes than CRC. Nevertheless, autopsy studies offer a unique opportunity to study the final distribution of metastases. During autopsy all intra-abdominal and intra-thoracic organs are extensively explored, revealing more metastases than would have been detected with imaging. This explains the high rate of distant lymph node metastases found in the autopsy cohort compared to the clinical cohort.

17% of cases (286/1679) in the autopsy cohort and 22% of cases (673/3092) in our clinical cohort had to be excluded because of non-documentation of the regional lymph node status, since there was no resection of the primary tumor. This might have caused bias in our patient selection. Moreover, there could have been variations in the quality of the autopsy examination and in the pathological examination of resected primary tumor specimens, which will have occurred in both groups. Therefore we do not expect a significant bias.

We cannot exclude the possibility that changes in the management of colorectal cancer between 1991-2010, would have had an influence on the metastatic patterns established at autopsy. The introduction of radiotherapy, chemotherapy, targeted therapy and surgery of metastatic lesions, might have shifted the presence of metastases to more uncommon sites. However, our main findings were validated in the population study with a narrower time frame.

This study shows that regional lymph node involvement in CRC is associated with a higher rate of peritoneal metastases and distant lymph node metastases. Our findings support the hypothesis that metastases to the liver and lung occur independently of lymphatic spread. Regional lymph node metastases function as a biomarker, i.e. as a sign of advanced disease, and seem only mechanisticly involved in the process of metastases in a small subgroup of patients with spread via the distant lymph nodes. Unfortunately, the presence of vascular invasion is grossly underreported in pathology reports, making a separate analysis for vascular invasion not possible. Therefore, our findings can only indirectly support the vascular pathway as a mechanism for development of common distant metastases, such as liver and lung metastases. However, the current recognition of extramural vascular invasion in the staging and treatment of colorectal cancer[19-21] seems justified.

References

1. Disibio, G. and S.W. French, *Metastatic patterns of cancers: results from a large autopsy study.* Arch Pathol Lab Med, 2008. **132**(6): p. 931-9.
2. Hess, K.R., et al., *Metastatic patterns in adenocarcinoma.* Cancer, 2006. **106**(7): p. 1624-33.
3. Weiss, L., et al., *Haematogenous metastatic patterns in colonic carcinoma: an analysis of 1541 necropsies.* J Pathol, 1986. **150**(3): p. 195-203.
4. Alitalo, A. and M. Detmar, *Interaction of tumor cells and lymphatic vessels in cancer progression.* Oncogene, 2012. **31**(42): p. 4499-508.
5. Budczies, J., et al., *The landscape of metastatic progression patterns across major human cancers.* Oncotarget, 2015. **6**(1): p. 570-83.
6. Hugen, N., et al., *Metastatic pattern in colorectal cancer is strongly influenced by histological subtype.* Ann Oncol, 2014. **25**(3): p. 651-7.
7. Casparie, M., et al., *Pathology databanking and biobanking in The Netherlands, a central role for PALGA, the nationwide histopathology and cytopathology data network and archive.* Cell Oncol, 2007. **29**(1): p. 19-24.
8. Fleming ID, C.S., Henson DE, Hutter RVP, Kennedy BJ, Murphy GP, O'Sullivan B, Sobin LH, Yarbro JW, editors, *American Joint Committe on Cancer (AJCC). AJCC Cancer Staging Manual*. 5th ed. 1997, Philadelphia: Lippincott JB.
9. Mekenkamp, L.J., et al., *Clinicopathological features and outcome in advanced colorectal cancer patients with synchronous vs metachronous metastases.* Br J Cancer, 2010. **103**(2): p. 159-64.
10. Coebergh, J.W., et al., *[Trends in incidence of cancer in southeast North Brabant and North Limburg during 1975-1986; report from the IKZ/SOOZ cancer registration (Integrated Cancer Center South/ Cooperative Organization Oncology Hospitals)].* Ned Tijdschr Geneeskd, 1990. **134**(15): p. 754-60.
11. Fritz AG, P.C., Jack A, Shanmugaratnam K, Sobin L, Parkin DM, Whelan S., *International classification of diseases for oncology, 3rd edition*, P.C. Fritz AG, Jack A, Shanmugaratnam K, Sobin L, Parkin DM, Whelan S., Editor. 2000, World Health Organization: Geneva.
12. Welch, J.P. and G.A. Donaldson, *The clinical correlation of an autopsy study of recurrent colorectal cancer.* Ann Surg, 1979. **189**(4): p. 496-502.
13. Jayne, D.G., et al., *Peritoneal carcinomatosis from colorectal cancer.* Br J Surg, 2002. **89**(12): p. 1545-50.
14. Segelman, J., et al., *Incidence, prevalence and risk factors for peritoneal carcinomatosis from colorectal cancer.* Br J Surg, 2012. **99**(5): p. 699-705.
15. van Gestel, Y.R., et al., *Metachronous peritoneal carcinomatosis after curative treatment of colorectal cancer.* Eur J Surg Oncol, 2014. **40**(8): p. 963-9.
16. van Santvoort, H.C., et al., *Peritoneal carcinomatosis in t4 colorectal cancer: occurrence and risk factors.* Ann Surg Oncol, 2014. **21**(5): p. 1686-91.
17. Koppe, M.J., et al., *Recent insights into the pathophysiology of omental metastases.* J Surg Oncol, 2014. **110**(6): p. 670-5.
18. Tulunay, G., et al., *Chylous ascites: analysis of 24 patients.* Gynecol Oncol, 2012. **127**(1): p. 191-7.
19. Betge, J., et al., *Intramural and extramural vascular invasion in colorectal cancer: prognostic significance and quality of pathology reporting.* Cancer, 2012. **118**(3): p. 628-38.
20. Chand, M., et al., *EMVI-positive stage II rectal cancer has similar clinical outcomes as stage III disease following pre-operative chemoradiotherapy.* Ann Oncol, 2014. **25**(4): p. 858-63.
21. Petersen, V.C., et al., *Identification of objective pathological prognostic determinants and models of prognosis in Dukes' B colon cancer.* Gut, 2002. **51**(1): p. 65-9.

Chapter 7

Perineural invasion is a strong prognostic factor in colorectal cancer – a systematic review

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Abstract

Perineural invasion (PNI) is a possible route for metastatic spread in various cancer types, including colorectal cancer. PNI is linked to poor prognosis, but systematic analyses are lacking. This study systematically reviews the frequency and impact of PNI in colorectal cancer.

A literature search was performed using PubMed database from inception to 1 January 2014. Data were analyzed using Review Manager 5.3. A quality assessment was performed based on modified REMARK criteria. Endpoints were local recurrence (LR), five year disease free survival (5yDFS), five year cancer specific survival (5yCSS) and five year overall survival (5yOS). Meta-analysis was performed in terms of risk ratios (RR) and hazard ratios (HR) with 95% confidence interval (95%CI).

In this meta-analysis 58 articles with 22900 patients were included. PNI was present in 18.2% of tumors. PNI is correlated with increased LR (RR 3.22, 95%CI 2.33-4.44), decreased 5yDFS (RR 2.35, 95%CI 1.66-3.31), 5yCSS (RR 3.61, 95%CI 2.76-4.72) and 5yOS (RR 2.09, 95%CI 1.68-2.61). In multivariate analysis PNI remains an independent prognostic factor for 5yDFS, 5yCSS and 5yOS (HR 2.35, 95%CI 1.97-3.08, HR 1.91, 95%CI 1.50-2.42, and HR 1.85, 95%CI 1.63-2.12, respectively).

We confirmed the strong impact of PNI for local recurrence and survival in colorectal cancer. The prognostic value of PNI is similar to that of well-established prognostic factors as depth of invasion, differentiation grade, lymph node metastases, lymphatic and extramural vascular invasion. Therefore, PNI should be one of the factors in the standardised reporting of colorectal cancer and might be considered a high-risk feature.

Introduction

The study of metastases formation is of utmost importance to find strategies to prevent future cancer deaths. There are several routes that allow the spread of tumor cells: in addition to direct growth, the cells can disseminate via the blood and lymph channels or grow along the nerves.[1] The latter is called perineural invasion (PNI). The tumor cells can grow within, around and through any of the three nerve layers. Tumor cells should by definition surround more than 33% of the nerve circumference for PNI.[1] PNI has been gaining increased recognition, however, its true value has not been established yet. There is a wide difference in the used definitions, in reported frequencies and impact on prognosis. High incidences of PNI are especially reported in pancreatic adenocarcinomas (98%), cholangiocarcinomas (75-85%), prostate (75%) and gastric adenocarcinomas (60%). In colorectal cancer (CRC) the incidence of PNI seems to be much lower.[1]

In CRC, well-established predictors of prognosis are depth of invasion, differentiation grade, presence of lymph node metastases, lymphangio-invasion and extramural vascular invasion. In the TNM 7th edition PNI was introduced as an accessory factor.[2] To establish the impact of PNI in CRC, we systematically reviewed the frequency and impact of PNI in CRC. The prognostic endpoints are local recurrence (LR), five year disease free survival (5yDFS), five year cancer specific survival (5yCSS) and five year overall survival (5yOS).

Materials and methods

Strategy for search of articles and selection criteria

A comprehensive literature search was performed using the PubMed database from inception to 1 January 2014, using the following keywords: “perineural” or “peri-neural” or “neural” or “nerve” or “nerves” and “invasion” or “Neoplasm Invasiveness”[Mesh] and “Peripheral Nerves”[Mesh] or “Peripheral Nervous System Neoplasms/secondary”[Mesh] or “Nervous System Neoplasms/secondary”[Mesh] and “Colorectal Neoplasms”[Mesh] or “colorectal” or “colon” or “rectum” or “rectal” and “cancer” or “carcinoma”. Additional searches were performed by manual cross-referencing and an expert in the field (IN) was consulted for additional articles that met the inclusion criteria.

Two independent investigators (SM, NK) reviewed each report for eligibility. Articles published in English and studies with at least 100 patients were selected. Studies with and without (neo)adjuvant treatment were included. In case of overlapping patient data, the study with most outcome data or longest follow-up was included

in this meta-analysis. In order to select for studies in which PNI was studied more systematically, studies with missing values of PNI of more than 10% were excluded.

Data extraction

For each study the number of patients in the PNI+ and the PNI- group were obtained, only extramural PNI was included in the PNI+ group. Data on tumor stage, neo-adjuvant therapy, LR, 5yDFS, 5yCSS and 5yOS were extracted. Data were entered in SPSS (SPSS for Windows, IBM SPSS Statistics 20, 2011) and Review Manager (RevMan) [Computer program]. Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012).

Quality assessment and risk of bias

A scale to assess the quality of the reporting of the studies was developed based on the REMARK guidelines, specifically looking at the reporting of PNI (Supplemental Digital Content, table 1: Quality of reporting scoring criteria used).[3, 4] Only studies with data on outcome were subjected to quality assessment. Scoring was performed by two independent investigators (NK, FS). In case of disagreement, a consensus score was achieved after discussion. The association between the quality of reporting and the RR/HR was analyzed with scatter plots and non-parametric correlation testing. Publication bias was assessed by looking at symmetry in funnel plots.

Statistical analysis

A meta-analysis was performed with all available studies on each endpoint in terms of risk ratios (RR) with 95%CI. Data of multivariate analyses were entered in terms of hazard ratios (HR) with 95%CI. A random effects model with inverse variance weighting of studies was used. Heterogeneity was assessed using a χ^2 test for heterogeneity with a p-value of <0.10 to show the presence of significant heterogeneity. In case of heterogeneity, sub-analyses were performed to identify the potential source of heterogeneity.

Results

Search results

A total of 439 potentially relevant studies were retrieved by the database search. 382 studies were excluded because they did not meet inclusion criteria (Figure 1). Cross-referencing and consulting an expert in the field resulted in one additional article that fulfilled the eligibility criteria. The remaining 58 articles, comprising 22900 patients, were included in the meta-analysis.[5-62]

19 studies investigated PNI in rectal cancer, ten studies in colon cancer and in 29 studies rectal and colon carcinomas were grouped together. Of the latter one study subdivided incidences of PNI in rectal and colon cancer. The 20 studies with data on PNI in rectal cancer contained 6942 patients; 11 colon cancer studies contained 4637 patients, and 28 studies with colorectal cancer incorporated 11321 cases. The main characteristics of the studies are shown in table 1. The mean number of patients included in the studies was 388 (range 100-2492).

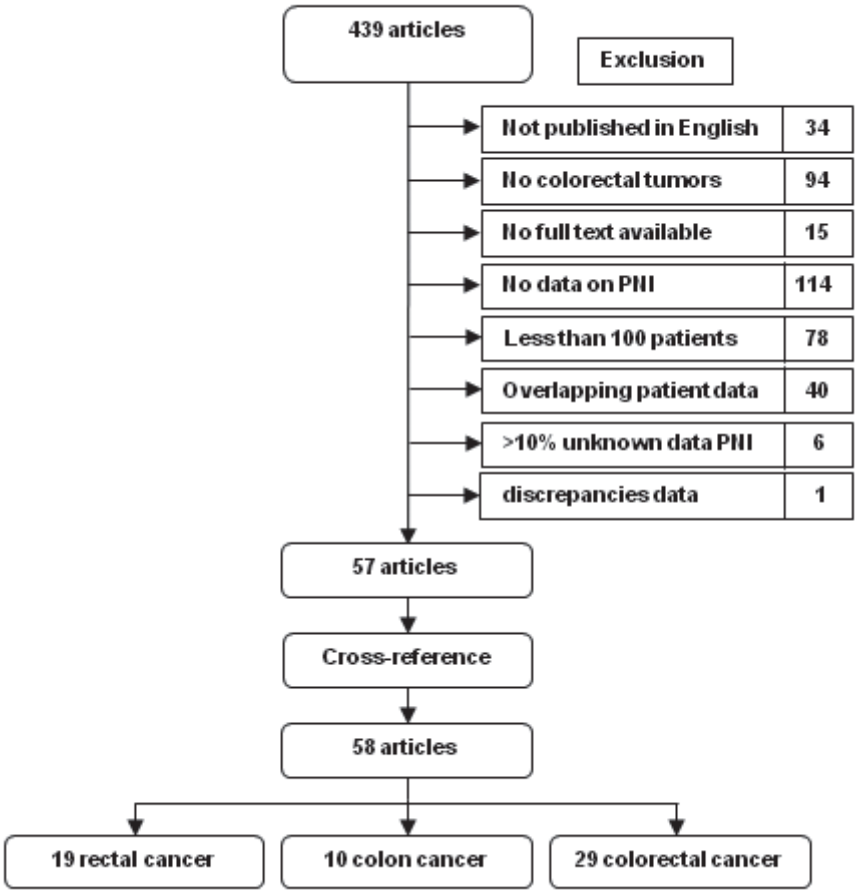


Figure 1. Flow chart of search strategy. Abbrevations: PNI:perineural invasion

Table 1A: Main characteristics of the articles on CRC

author	country	time-frame	study design	stage	neoadjuvant RT	n	%PNI
Allard MA	France	1998-2008	RSCS	II-III	no	117	21.4%
Bamias A	Greece	1989-1997	RMCS	II-III	nm	499	7.8%
Barresi V	Italy	Nm	RSCS	I-IV	no	152	21.7%
Bellis D	Italy	1988-1989	RSCS	nm	nm	160	31.3%
Bouassida M	Tunesia	2000-2010	RCCS	I-IV	yes, ns	280	32.9%
Chang DT	USA	2000-2010	RCCS	I-IV	nm	128	18.8%
Choi PW	Korea	1989-2004	RSCS	I	no	168	0.6%
Cohn KH	USA	1988-1992	RSCS	I-IV	nm	104	8.7%
da Fonseca LM	Brazil	2001-2010	RSCS	I-IV	nm	653	23.4%
Dogan L	Turkey	1999-2006	RSCS	II-III	no	116	5.2%
Galindo Gallego M	Spain	1982-1991	RSCS	I-III	no	126	10.3%
Gray KD	USA	1990-1999	RMCS	I-IV	nm	213	12.7%
Guerra A	Spain	Nm	RSCS	I-IV	nm	108	11.1%
Huang CW	Taiwan	2002-2008	RSCS	I-IV	nm	1197	37.3%
Huh JW	Korea	1997-2009	RSCS	I-III	no	1732	22.7%
Ianoşi G	Romenia	Nm	RSCS	I-IV	nm	273	8.1%
Kang H	Korea	1996-1999	RMCS	I-IV	nm	301	26.3%
Kim JY	Korea	1998-2003	RSCS	I-IV	no	292	23.6%
Liebig C	USA	1995-2000	RSCS	I-IV	nm	249	22.1%
Lin M	China	2005-2008	RSCS	I-IV	nm	123	20.3%
Oñate-Ocaña LF	Mexico	1983-1998	RSCS	I-II	no	124	41.9%
Pagès F	France	1986-2004	RSCS	I-IV	nm	959	10.3%
Shiozawa J	Japan	1996-1998	RSCS	I-IV	nm	115	19.1%
Ting WC	China	2001-2007	RSCS	I-IV	nm	282	17.0%
Ueno H	Japan	1999-2004	RMCS	I-III	no	2492	12.6%
Viana Lde S	Brazil	2006-2009	RSCS	I-IV	no	114	7.0%
Yun HR	Korea	1994-2004	RSCS	IV	no	127	12.6%
						11321	18.5%

Abbrevations: RSCS: retrospective single center study; RMCS: retrospective multi center study; RCCS: retrospective case control study; RT: radiotherapy; nm: not mentioned; ns: not specified; n: number of patients; PNI: perineural invasion.

Table 1B. Main characteristics of articles on rectal cancer

author	country	time-frame	study design	stage	neoadjuvant RT	n	%PNI
Bentzen SM	Denmark	1979-1985	RMCS	II-III	no	494	26.1%
Ceyhan GO	Germany	1990-2002	RSCS	II-III	yes, 53.0% CRT 45Gy/5FU	275	18.9%
Chandrasinghe PC	Sri Lanka	1996-2010	RSCS	I-IV	yes, 33.6% CRT (ns)	226	11.1%
Dresen RC	Netherlands	1994-2006	RCCS	I-III	yes, 53.4% RT (5x5Gy/50Gy)	277	7.2%
Guillem JG	USA	1988-2002	RSCS	0-IV	yes, 100% CRT 50Gy/5FU	297	9.1%
Horn A	Norway	Nm	RS	I-III	yes, 49.6% RT (ns)	254	35.4%
Kim JS	Korea	2001-2007	RSCS	nm	yes, 65.2% CRT 50Gy/5FU	797	26.0%
Knudsen JB	Denmark	1968-1980	RSCS	I-IV	no	673	34.9%
Krebs B	Slovenia	1998-2003	RSCS	0-IV	nm	247	12.6%
Law WL	Hong Kong	2000-2006	RSCS	II-III	yes, 12.8% CRT (ns)	421	13.5%
Lim JW	Singapore	1999-2007	RSCS	I-III	no	261	18.4%
Peng J	China	1996-2004	RSCS	II	no	173	24.3%
Peng J	China	1992-2005	RSCS	I, III	no	124	9.7%
Poeschl EM*	Austria	Nm	RSCS	I-IV	no	149	18.1%
Rullier A	France	Nm	RSCS	II-III	yes, 100% RT 45Gy, 77% CRT 5FU	200	15.5%
Seefeld PH	USA	1935-1936	RSCS	I-IV	no	100	30.0%
Shirouzu K	Japan	1982-1992	RSCS	I-IV	nm	501	20.0%
Silberfein EJ	USA	1993-2003	RSCS	I-III	yes, 88.8% CRT 45Gy/5FU	304	6.3%
Sitzler PJ	Singapore	1989-1996	RSCS	nm	no	805	24.6%
Ueno H	Japan	1981-1995	RSCS	II-III	no	364	14.3%
Total						6942	20.6%

Abbrevations: RSCS: retrospective single center study; RS: randomized study; RCCS: retrospective case control study; RMCS: retrospective multi center study; RT: radiotherapy; CRT: chemoradiotherapy; nm: not mentioned; ns: not specified; n: number of patients; PNI: perineural invasion; n.p: not performed; *seperate data on colon and rectal cancer

Table 1C. Main characteristics of articles on colon cancer

author	country	time-frame	study design	stage	n	%PNI
Desolneux G	USA	1976-1989	RSCS	I-II	362	4.4%
Jee SH	Korea	1993-2006	RSCS	II	363	14.3%
Lennon AM	Ireland	1992-1997	RSCS	II	118	11.0%
Liebl F	Germany	1990-2005	RSCS	I-IV	673	31.2%
Oh	Korea	1998-2003	RSCS	II-III	340	13.2%
Peng SL	Australia	1999-2007	RMCS	II	458	3.7%
Poeschl EM*	Austria	Nm	RSCS	I-IV	232	13.4%
Stor Z	Slovenia	1994-2000	RSCS	II	191	2.1%
Tanaka M	Japan	1981-1993	RSCS	II	138	9.4%
Weiser MR	USA	1990-2000	RSCS	I-III	1320	6.0%
Wied U	Denmark	1970-1980	RSCS	I-IV	442	39.1%
					4637	14.1%

Abbrevations: nm: not mentioned; RMCS: retrospective multi center study; RSCS: retrospective single center study; n: number of patients; PNI: perineural invasion; *seperate data on colon and rectal cancer

Quality of reporting of the included studies

Studies with outcome data (n=31) were subjected to quality assessment, focussed on the quality of reporting of PNI (Supplemental Digital Content, table 2: Reporting scores per article, 0:no; 1:yes; na:not applicable). The mean percentage of items reported was 61.8% (range 17.7-84.2%). Only 4 studies reported less than 50% of required items, 22 studies reported 50-75% of required items and 5 studies reported >75% of items (Supplemental Digital Content, Figure 1: Overview of the percentage of items reported).

Frequency of PNI

The incidence of PNI was 18.2% in the overall cohort, 20.6% in rectal cancer and 14.1% in colon cancer studies (table 1). The percentage of PNI was not significantly different between studies that re-examined pathologic slides (25 studies; 8483 patients) and studies that extracted PNI from pathology reports (33 studies; 14417 patients) (19.0% vs. 18.1%, p=0.10).

The frequency of PNI was stage dependent, with very low incidence in stage I (0.2%, total 412 cases). Tumors without lymph node metastases (stage I and II together) showed PNI in 9.5% (total 5353 cases), compared to 26.3% in stage III tumors (total 1840 cases) and 36.6% in tumors with distant metastases at the time

of presentation (total 287 cases)(Figure 2A). These differences could be explained by both an increased risk of PNI with increasing T-stage (total 4818 cases)(Figure 2B) [11, 47, 51, 53, 57, 58] and N-stage (total 3512 cases)(Figure 2C).[11, 47, 57, 58] PNI occurred more often in tumors with poor differentiation (23.8%, total 416 cases) compared to well differentiated (6.9%, total 1441 cases) and moderately differentiated tumors (17.8%, total 1849 cases) (Figure 2D).[11, 36, 47, 57, 58]

Association between PNI and lymphatic and venous invasion

The association between PNI, lymphatic and venous invasion was investigated in four studies.[45, 47, 51, 58] One study[45] did not differentiate between vascular and lymphatic invasion and detected PNI in 19.6% of the tumors without lymphovascular invasion (LVI) compared to PNI in 52% of the tumors with LVI ($p<0.001$). When combining the other three studies, PNI was observed in 4.6% of the tumors without (total 1408 cases) and in 17.6% of the tumors with lymphatic invasion (total 2319 cases). PNI occurred in 4.4% of the tumors without venous invasion (total 1565 cases), compared to 18.7% in tumors with venous invasion (total 2160 cases). Thus, PNI positive tumors more often have lymphatic invasion compared to PNI negative tumors (RR 1.92, 95% CI 1.36-2.72, $p<0.001$ (Supplemental Digital Content, Figure 2A: Association between PNI and lymphatic invasion)), and more often have venous invasion (RR 1.92, 95% CI 1.46-2.51, $p<0.001$ (Supplemental Digital Content, Figure 2B: Association between PNI and venous invasion)). In both analyses significant heterogeneity was observed ($\chi^2=50.05$, df 2 ($p<0.001$), $I^2=96\%$ and $\chi^2=55.21$, df 2 ($p<0.001$), $I^2=96\%$), respectively), but sub-analyses were not possible due to the limited number of studies.

Effect of neo-adjuvant treatment on PNI

Approximately half of the patients with rectal cancer received some form of neo-adjuvant treatment.[11, 12, 19, 23, 24, 30, 34, 48, 52] In two studies all patients received neo-adjuvant (chemo-)radiation (497 cases).[23, 48] In seven studies some patients received neo-adjuvant treatment (percentages of treated patients varying between 12.8% and 88.8%), which varied between short schedule radiotherapy, long schedule radiotherapy or combined chemo- and radiotherapy (total 2575 cases, 1224 with and 1351 without radiotherapy). Two studies did not mention neo-adjuvant treatment (748 cases).[33, 51] In eight studies no neo-adjuvant treatment was given (2994 cases).[9, 32, 38, 44, 45, 49, 53, 57] In the neo-adjuvant treatment group 528 of 3072 patients (17.2%) showed PNI. Of the 2994 patients who did not receive neo-adjuvant therapy, 746 showed PNI (24.9%)($p<0.001$).

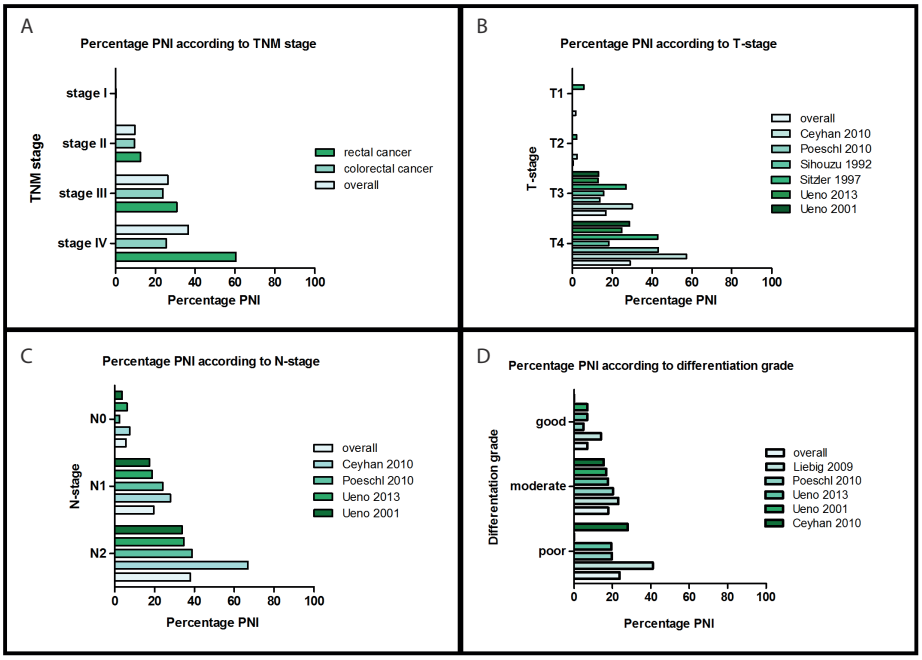
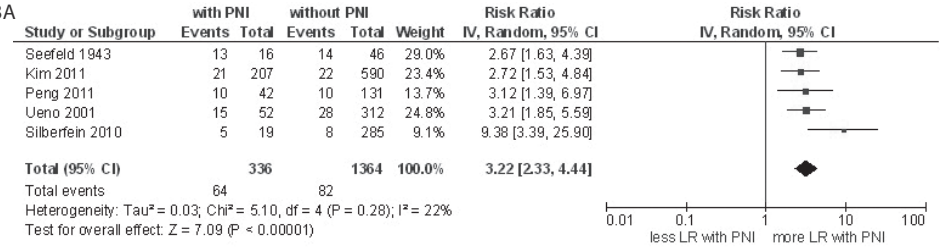


Figure 2. The frequency of PNI is stage dependent. A) Percentage of PNI in different TNM-stages, subdivided into rectal and colorectal cancer, 15 studies with 4954 patients included.[9, 11, 14, 28, 35, 36, 45-47, 51, 54, 55, 57, 58, 62] B) Percentage of PNI according to T-stage extracted from six studies with 4818 patients. C) Percentage of PNI according to N-stage extracted from four studies with 3512 patients. D) Percentage PNI according to differentiation grade extracted from five studies with 3706 patients. Abbrevations: PNI:perineural invasion

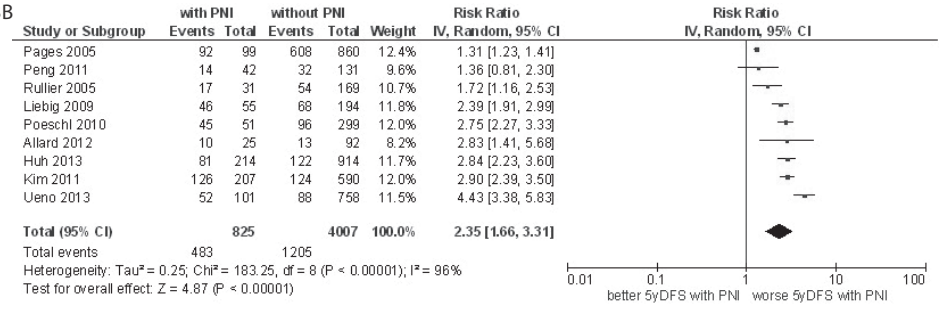
Effect of PNI on local recurrence

The influence of PNI on LR could be extracted from five studies comprising 1700 patients.[30, 45, 49, 52, 57] The presence of PNI is associated with increased risk of LR with a RR of 3.22 (95%CI 2.33-4.44, p<0.001) (Figure 3A). No heterogeneity was observed (χ^2=5.10, df=4 (p=0.28); I^2=22%), and there was no indication of publication bias. The percentage of items reported ranged from 50% to 83.3%, and this quality indicator did not correlate with the magnitude of RR (Spearman r=0.53, p=0.35). Furthermore, studies in which PNI was extracted from pathology reports showed similar RR compared to studies with specific review of PNI (6.05 vs. 3.00, p=0.31).

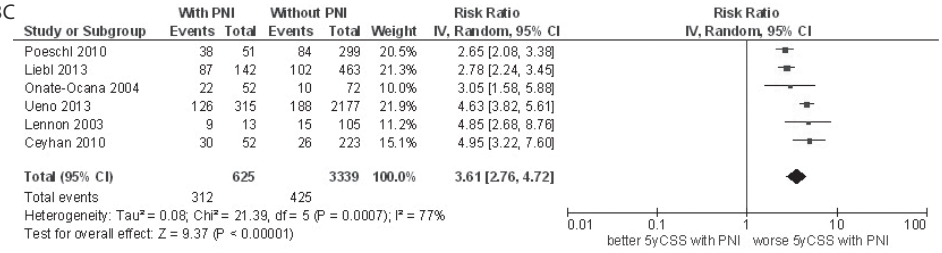
3A



3B



3C



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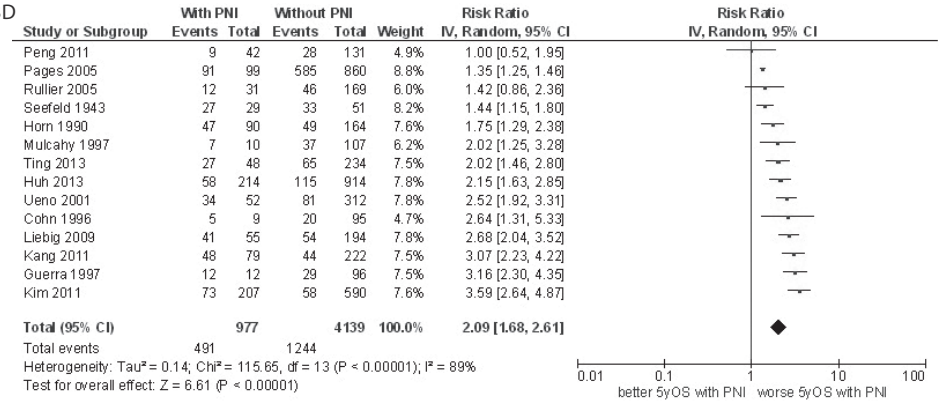


Figure 3. The impact of PNI on outcome in univariate analysis. A) Impact of PNI on LR. B) Impact of PNI on 5yDFS. C) Impact of PNI on 5yCSS. D) Impact of PNI on 5yOS. Abbrevations: PNI:perineural invasion; IV:inverse variance; CI:confidence interval; LR:local recurrence; 5yDFS:5-year disease free survival; 5yCSS:5year cancer specific survival; 5yOS:5year overall survival

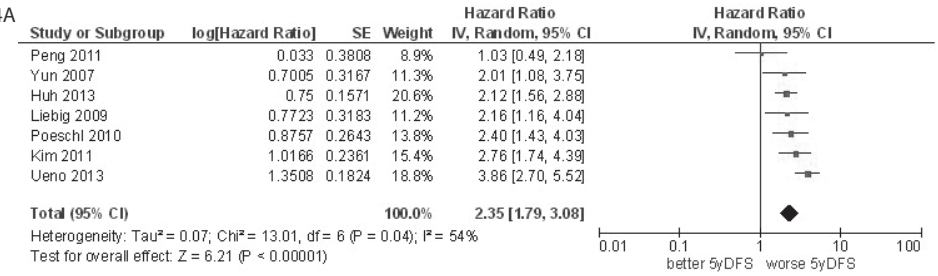
Two studies provided data on multivariate analysis, in both PNI is an independent prognostic factor for LR.[45, 57] A study by Ueno et al. shows that PNI is an independent risk factor for LR with an OR of 5.4 (95%CI 2.3-12.8).[57], which is comparable to lymph node involvement (OR 3.4, 95%CI 1.3-8.8). Patients with positive resection margins were excluded. Peng et al. found that PNI-positivity was the only independent risk factor for LR (HR 2.70, 95%CI 1.03-7.06, p=0.04).[45] Tumor grade, circumferential resection margin, LVI and number of sampled lymph nodes were not associated with local recurrence in multivariate analysis. One other study performed a multivariate analysis, but did not provide separate data.[30]

Effect of PNI on disease free survival

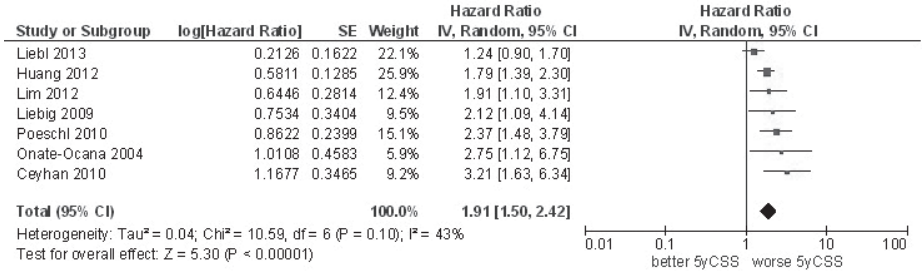
Data on the impact of PNI on the 5yDFS were available from nine studies with in total 4832 patients.[5, 26, 30, 36, 43, 45, 47, 48, 58] The 5yDFS decreased in the presence of PNI (RR 2.35, 95%CI 1.66-3.31, p<0.001) (Figure 3B). Significant heterogeneity was observed between the studies (χ^2=183.25, df=8 (p<0.001); I^2=96%). Sample size, time-frame, neo-adjuvant therapy, location of the primary tumor and TNM stage did not explain the heterogeneity. There was no indication of publication bias. The percentage of items reported in the articles ranged from 47.4% to 84.2%, and this quality indicator did not correlate with the magnitude of RR (Spearman r=-0.28, p=0.46). No difference was observed between studies with PNI from pathology reports and studies with specific review of PNI (2.35 vs. 2.58, p=0.76).

Multivariate analysis was available in 7 studies comprising 5951 patients, confirming a decreased 5yDFS in the presence of PNI (HR 2.35, 95%CI 1.79-3.08, p<0.001) (Figure 4A). Significant heterogeneity was observed between the studies (χ^2=13.01, df=6 (p=0.04); I^2=54%). We investigated study size, time-frame, neo-adjuvant therapy, location of the primary tumor and TNM stage, but these factors did not explain the heterogeneity. There was no indication of publication bias. The percentage of items reported varied from 38.9% to 84.2%, and did not correlate with the magnitude of HR (Spearman r=-0.05, p=0.91). Studies in which PNI was extracted from pathology reports showed similar HR compared to studies with specific review of PNI (2.30 vs. 2.36, p=0.93). Other independent prognostic factors for 5yDFS in multivariate analysis were T-stage, N-stage and LVI. The HR of these factors were comparable with that of PNI (T-stage: combined HR of 3.13 (95%CI 2.05-4.79)[26, 47], N-stage: combined HR of 2.22 (95%CI 1.72-2.87)[26, 58, 62], LVI: combined HR of 1.81 (95%CI 1.44-2.27)[26, 47, 58]).

4A



4B



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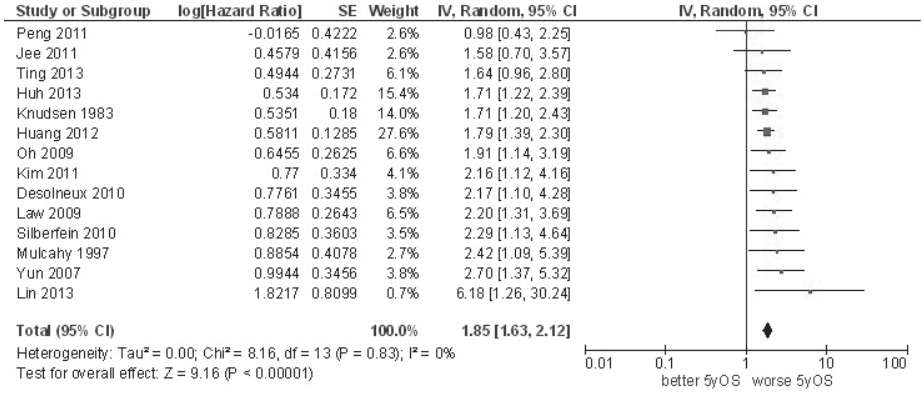


Figure 4. The impact of PNI on outcome in multivariate analysis. A) Impact of PNI on 5yDFS. B) Impact of PNI on 5yCSS. C) Impact of PNI on 5yOS. Abbrevations: PNI:perineural invasion; SE:standard error; IV:inverse variance; CI:confidence interval; 5yDFS:5-year disease free survival; 5yCSS:5year cancer specific survival; 5yOS:5year overall survival

Effect of PNI on cancer specific survival

The impact of PNI on 5yCSS could be extracted from six studies with in total 3964 patients.[11, 35, 37, 42, 47, 58] The presence of PNI is associated with worse 5yCSS (RR 3.61, 95%CI 2.76-4.72, p<0.001)(Figure 3C). Significant heterogeneity was observed (χ^2=21.39, df=5 (p<0.001); I^2=77%). We investigated study size, time-frame, location of the primary tumor and TNM stage. These factors did not explain the heterogeneity. There was no indication of publication bias. With respect to the quality of the articles, the percentage of items reported ranged from 50% to 83.3%, and this did not correlate with the magnitude of RR (Spearman r=0.23, p=0.66). PNI was rescored in all studies with univariate data on 5yCSS.

Multivariate analysis was performed in seven studies comprising 3160 patients, confirming a decreased 5yCSS in the presence of PNI (HR 1.91, 95%CI 1.50-2.42, p<0.001)(Figure 4B).[11, 25, 36-38, 42, 47] No heterogeneity was observed (χ^2=10.59, df=6 (p=0.10); I^2=43%). There was no indication of publication bias. The percentage of items reported varied from 52.6% to 84.2% and this did not correlate with the magnitude of HR (Spearman r=0.14, p=0.78). Studies in which PNI was extracted from pathology reports showed similar HR compared to studies with specific review of PNI (1.85 vs. 2.34, p=0.41).

Other independent prognostic factors for 5yCSS in multivariate analysis were T-stage, N-stage, LVI and differentiation grade. The HR of these factors were comparable with that of PNI (T-stage: combined HR of 3.66 (95%CI 2.21-6.06)[11, 37, 38, 47], N-stage: combined HR of 2.59 (95%CI 1.59-4.21)[11, 37, 38], LVI: combined HR of 1.81 (95%CI 1.44-2.27)[25, 37, 47], differentiation grade: combined HR of 1.51 (95%CI 1.08-2.11)[25, 36, 37, 42, 47]).

Effect of PNI on overall survival

The impact of PNI on 5yOS was evaluated in 14 studies with in total 5116 patients.[15, 22, 24, 26, 29, 30, 36, 40, 43, 45, 48, 49, 56, 57] The 5yOS decreased in the presence of PNI (RR 2.09, 95%CI 1.68-2.61, p<0.001) (Figure 3D). Significant heterogeneity was observed (χ^2=115.65, df=13 (p<0.001); I^2=89%). We investigated the factors study size, time-frame, neo-adjuvant therapy, location of the primary tumor and TNM stage. These factors did not explain the heterogeneity. There was no indication of publication bias. The percentage of items reported varied from 17.7% to 84.2%, and did not correlate with the magnitude of RR (Spearman r=-0.15, p=0.61). Studies in which PNI was extracted from pathology reports showed similar RR compared to studies with specific review of PNI (2.49 vs. 1.99, p=0.25).

Multivariate analysis was performed in 14 studies comprising 7011 patients.[17, 25, 26, 28, 30, 32, 34, 39-41, 45, 52, 56, 62] However, four studies did not provide separate data.[20, 46, 48, 61] The presence of PNI is associated with decreased 5yOS, combined hazard ratio of

1.85 (95%CI 1.63-2.12, p<0.001)(Figure 4C). No heterogeneity was observed (χ^2=8.16, df=13 (p=0.83); I^2=0%). There was no indication of publication bias. The percentage of items reported ranged from 38.9% to 83.3% and this did not correlate with the magnitude of HR (Spearman r=-0.39, p=0.17). Studies in which PNI was extracted from pathology reports showed similar HR compared to studies with specific review of PNI (2.39 vs. 1.70, p=0.40). Other independent prognostic factors for 5yOS were T-stage, N-stage, LVI and differentiation grade. The HR of these factors were comparable with that of PNI (T-stage: HR of 2.08 (95%CI 1.46-2.95)[17, 26, 28], N-stage: combined HR of 1.97 (95%CI 1.63-2.37)[26, 30, 34, 39, 41, 56, 62], LVI: combined HR of 1.76 (95%CI 1.52-2.03)[17, 25, 26, 28, 32, 39, 41, 52, 56], differentiation grade: combined HR of 1.76 (95% CI 1.25-2.47)[25, 34, 56]).

Discussion

The overall incidence of PNI found in this meta-analysis was 18.2%, which is lower than the 33% found in a previous review.[1] That review included four studies (478 patients in total) that had PNI as their main focus, and in two of these studies immunohistochemistry was used to detect PNI.[8, 63] In our meta-analysis both studies which re-examined pathologic slides with a specific focus on PNI and studies that extracted PNI from pathology reports were included. Interestingly, the percentage of PNI was not significantly different between studies that re-examined pathologic slides and studies that extracted PNI from pathology reports, suggesting that this biomarker is well reported in daily practice. In studies that re-examined PNI, the method of scoring was often not clearly described. Definitions of PNI and the number of scorers were often lacking. However, it is clear that PNI is relatively uncommon in CRC compared to other cancer types. One explanation for this difference might be a lower neural density in colorectal tissue compared to other tissues. Likewise, differences in the expression of neurotrophins, chemokines, proteinases and their receptors may be involved.[1, 50, 64]

Our meta-analysis shows increased PNI rates in rectal cancer (20.6%) compared to colon cancer (14.1%),[36, 47, 65] which might be explained by the fact that the rectum is surrounded by many autonomic nerve plexuses. The colon is intraperitoneal and lacks an external plexus and thus has less innervation than the rectum. Moreover, more extensive examination of the mesorectal fat in rectal carcinoma for investigating CRM involvement may increase detection of PNI.

In our meta-analysis we tried to evaluate the influence of neo-adjuvant treatment on PNI. Since not all studies reported data on neo-adjuvant treatment, the effect of radiotherapy on PNI was difficult to establish. It seems that tumors without neo-adjuvant therapy have a higher incidence of PNI (24.9% versus 17.2%). However, in the current analyses different treatment schedules were compared. One randomized

controlled trial compared the effects of neo-adjuvant chemoradiation (45Gy/5-FU) directly.[11] They showed 19% PNI in tumors after chemoradiation compared to 32% in tumors without treatment (p=0.01).

Differences in reporting of published studies hampered our investigation. Some studies had to be excluded because they did not fulfil our criteria of outcome, for example they used 3 or 8 year survival or local and distant recurrence were grouped together. A more uniform reporting or adherence to the REMARK criteria would have given more comparable data. The overall quality of reporting was moderate, with a mean percentage of reported items of 61%. However, some studies did not give criteria for PNI positivity or the estimated effect of PNI in uni- or multivariate analysis was not given. Moreover, PNI was not re-examined in many studies and therefore the 'real' percentage of PNI could differ. However, this effect might be small, given the lack of difference in outcome between studies in which PNI was extracted from pathology reports compared to the studies with specific review of PNI.

We confirmed and quantified the strong negative prognostic impact of PNI for recurrence and survival in CRC. The largest number of studies and patients could be included in the analyses for OS. Here the HR was 1.85 (95%CI 1.63-2.12, p<0.001), and most comparable to T-stage, N-stage, LVI and differentiation grade. The biggest effect was observed for DFS. Here the HR was 2.35 (95%CI 1.79-3.08, p<0.001), an effect comparable to T-stage, N-stage and LVI. It should be noted that we observed significant heterogeneity in four out of seven pooled analyses, which we could not resolve by sub-analyses, but the direction of the effect was consistent over studies.

In summary, our meta-analysis shows that PNI is a pathologic feature in CRC with a strong impact on prognosis. The impact of PNI on prognosis is similar to well established prognostic factors as depth of invasion, presence of lymph node metastases, lymphatic invasion, vascular invasion and differentiation grade. Therefore, PNI should be one of the factors in the standardised reporting of CRC and might be considered a high-risk feature.

References

1. Liebig, C., et al., *Perineural invasion in cancer: a review of the literature.* Cancer, 2009. **115**(15): p. 3379-91.
2. Sobin, L.H., et al., *TNM classification of malignant tumours*. 7th ed. 2010, Chichester, West Sussex, UK ; Hoboken, NJ: Wiley-Blackwell. xx, 309 p.
3. Knijn, N., F. Simmer, and I.D. Nagtegaal, *Recommendations for reporting histopathology studies: a proposal.* Virchows Arch, 2015.
4. McShane, L.M., et al., *Reporting recommendations for tumor marker prognostic studies.* J Clin Oncol, 2005. **23**(36): p. 9067-72.
5. Allard, M.A., et al., *Linear quantification of lymphoid infiltration of the tumor margin: a reproducible method, developed with colorectal cancer tissues, for assessing a highly variable prognostic factor.* Diagn Pathol, 2012. **7**: p. 156.
6. Bamias, A., et al., *Prognostic factors in patients with colorectal cancer receiving adjuvant chemotherapy or chemoradiotherapy: a pooled analysis of two randomized studies.* Int J Gastrointest Cancer, 2005. **36**(1): p. 29-38.
7. Barresi, V., et al., *Histologic grading based on counting poorly differentiated clusters in preoperative biopsy predicts nodal involvement and pTNM stage in colorectal cancer patients.* Hum Pathol, 2014. **45**(2): p. 268-75.
8. Bellis, D., V. Marci, and G. Monga, *Light microscopic and immunohistochemical evaluation of vascular and neural invasion in colorectal cancer.* Pathol Res Pract, 1993. **189**(4): p. 443-7.
9. Bentzen, S.M., et al., *A regression analysis of prognostic factors after resection of Dukes' B and C carcinoma of the rectum and rectosigmoid. Does post-operative radiotherapy change the prognosis?* Br J Cancer, 1988. **58**(2): p. 195-201.
10. Bouassida, M., et al., *Histopathologic characteristics and short-term outcomes of colorectal cancer in young Tunisian patients: one center's experience.* Pan Afr Med J, 2012. **12**: p. 10.
11. Ceyhan, G.O., et al., *The severity of neural invasion is a crucial prognostic factor in rectal cancer independent of neoadjuvant radiochemotherapy.* Ann Surg, 2010. **252**(5): p. 797-804.
12. Chandrasinghe, P.C., et al., *Pre-operative hypoalbuminaemia predicts poor overall survival in rectal cancer: a retrospective cohort analysis.* BMC Clin Pathol, 2013. **13**: p. 12.
13. Chang, D.T., et al., *Clinicopathologic and molecular features of sporadic early-onset colorectal adenocarcinoma: an adenocarcinoma with frequent signet ring cell differentiation, rectal and sigmoid involvement, and adverse morphologic features.* Mod Pathol, 2012. **25**(8): p. 1128-39.
14. Choi, P.W., et al., *Risk factors for lymph node metastasis in submucosal invasive colorectal cancer.* World J Surg, 2008. **32**(9): p. 2089-94.
15. Cohn, K.H., et al., *The significance of allelic deletions and aneuploidy in colorectal carcinoma. Results of a 5-year follow-up study.* Cancer, 1997. **79**(2): p. 233-44.
16. da Fonseca, L.M., et al., *Colorectal carcinoma in different age groups : a histopathological analysis.* Int J Colorectal Dis, 2012. **27**(2): p. 249-55.
17. Desolneux, G., et al., *Prognostic factors in node-negative colorectal cancer: a retrospective study from a prospective database.* Int J Colorectal Dis, 2010. **25**(7): p. 829-34.
18. Dogan, L., et al., *Characteristics and risk factors for colorectal cancer recurrence.* J BUON, 2010. **15**(1): p. 61-7.
19. Dresen, R.C., et al., *Local recurrence in rectal cancer can be predicted by histopathological factors.* Eur J Surg Oncol, 2009. **35**(10): p. 1071-7.
20. Galindo Gallego, M., et al., *Vascular enumeration as a prognosticator for colorectal carcinoma.* Eur J Cancer, 2000. **36**(1): p. 55-60.
21. Gray, K.D., et al., *Do adverse histopathologic findings in colorectal cancer patients explain disparate outcomes?* J Natl Med Assoc, 2006. **98**(3): p. 348-51.

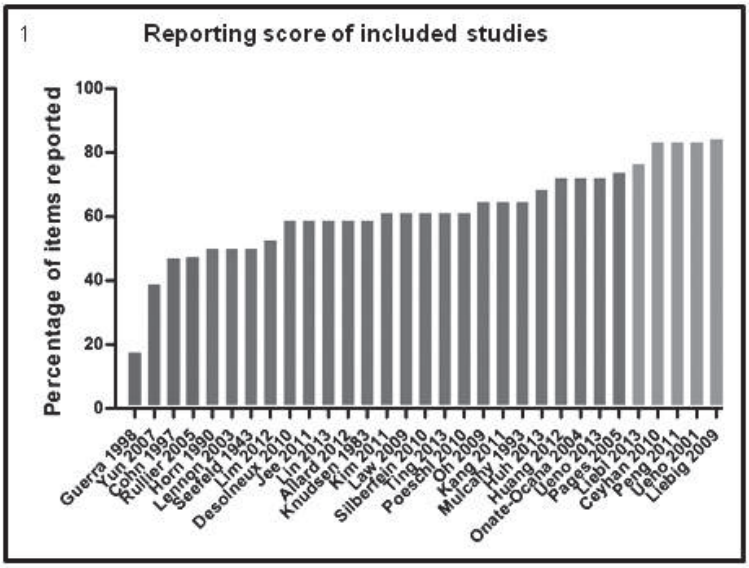
22. Guerra, A., et al., *Multivariate analysis of prognostic factors in resected colorectal cancer: a new prognostic index.* Eur J Gastroenterol Hepatol, 1998. **10**(1): p. 51-8.
23. Guillem, J.G., et al., *Long-term oncologic outcome following preoperative combined modality therapy and total mesorectal excision of locally advanced rectal cancer.* Ann Surg, 2005. **241**(5): p. 829-36; discussion 836-8.
24. Horn, A., O. Dahl, and I. Morild, *Venous and neural invasion as predictors of recurrence in rectal adenocarcinoma.* Dis Colon Rectum, 1991. **34**(9): p. 798-804.
25. Huang, C.W., et al., *The impact on clinical outcome of high prevalence of diabetes mellitus in Taiwanese patients with colorectal cancer.* World J Surg Oncol, 2012. **10**: p. 76.
26. Huh, J.W., et al., *Factors predicting long-term survival in colorectal cancer patients with a normal preoperative serum level of carcinoembryonic antigen.* J Cancer Res Clin Oncol, 2013.
27. Ianosi, G., et al., *Histopathological factors as predictors for survival in colon and rectal cancers.* Rom J Morphol Embryol, 2008. **49**(3): p. 365-9.
28. Jee, S.H., et al., *Effectiveness of Adjuvant Chemotherapy with 5-FU/Leucovorin and Prognosis in Stage II Colon Cancer.* J Korean Soc Coloproctol, 2011. **27**(6): p. 322-8.
29. Kang, H., et al., *Loss of E-cadherin and MUC2 expressions correlated with poor survival in patients with stages II and III colorectal carcinoma.* Ann Surg Oncol, 2011. **18**(3): p. 711-9.
30. Kim, J.S., et al., *Prognostic significance of distribution of lymph node metastasis in advanced mid or low rectal cancer.* J Surg Oncol, 2011. **104**(5): p. 486-92.
31. Kim, J.Y., et al., *Prognostic significance of epidermal growth factor receptor and vascular endothelial growth factor receptor in colorectal adenocarcinoma.* APMIS, 2011. **119**(7): p. 449-59.
32. Knudsen, J.B., et al., *Venous and nerve invasion as prognostic factors in postoperative survival of patients with resectable cancer of the rectum.* Dis Colon Rectum, 1983. **26**(9): p. 613-7.
33. Krebs, B., et al., *Prognostic value of additional pathological variables for long-term survival after curative resection of rectal cancer.* World J Gastroenterol, 2006. **12**(28): p. 4565-8.
34. Law, W.L., et al., *Comparison of outcome of open and laparoscopic resection for stage II and stage III rectal cancer.* Ann Surg Oncol, 2009. **16**(6): p. 1488-93.
35. Lennon, A.M., et al., *Peritoneal involvement in stage II colon cancer.* Am J Clin Pathol, 2003. **119**(1): p. 108-13.
36. Liebig, C., et al., *Perineural invasion is an independent predictor of outcome in colorectal cancer.* J Clin Oncol, 2009. **27**(31): p. 5131-7.
37. Liebl, F., et al., *The severity of neural invasion is associated with shortened survival in colon cancer.* Clin Cancer Res, 2013. **19**(1): p. 50-61.
38. Lim, J.W., et al., *Close distal margins do not increase rectal cancer recurrence after sphincter-saving surgery without neoadjuvant therapy.* Int J Colorectal Dis, 2012. **27**(10): p. 1285-94.
39. Lin, M., et al., *Elevated pre-treatment levels of high sensitivity C-reactive protein as a potential prognosticator in patients with colorectal cancer.* Exp Ther Med, 2013. **6**(6): p. 1369-1374.
40. Mulcahy, H.E., et al., *Identifying stage B colorectal cancer patients at high risk of tumor recurrence and death.* Dis Colon Rectum, 1997. **40**(3): p. 326-31.
41. Oh, S.Y., et al., *Contiguous invasion per se does not affect prognosis in colon cancer.* J Surg Oncol, 2009. **99**(1): p. 71-4.
42. Onate-Ocana, L.F., et al., *Identification of patients with high-risk lymph node-negative colorectal cancer and potential benefit from adjuvant chemotherapy.* Jpn J Clin Oncol, 2004. **34**(6): p. 323-8.
43. Pages, F., et al., *Effector memory T cells, early metastasis, and survival in colorectal cancer.* N Engl J Med, 2005. **353**(25): p. 2654-66.
44. Peng, J., et al., *Oncological outcome of T1 rectal cancer undergoing standard resection and local excision.* Colorectal Dis, 2011. **13**(2): p. e14-9.
45. Peng, J., et al., *Perineural invasion in pT3N0 rectal cancer: the incidence and its prognostic effect.* Cancer, 2011. **117**(7): p. 1415-21.

46. Peng, S.L., et al., *Conventional adverse features do not predict response to adjuvant chemotherapy in stage II colon cancer.* ANZ J Surg, 2013.
47. Poeschl, E.M., et al., *Perineural invasion: correlation with aggressive phenotype and independent prognostic variable in both colon and rectum cancer.* J Clin Oncol, 2010. **28**(21): p. e358-60; author reply e361-2.
48. Rullier, A., et al., *Impact of colloid response on survival after preoperative radiotherapy in locally advanced rectal carcinoma.* Am J Surg Pathol, 2005. **29**(5): p. 602-6.
49. Seefeld, P.H. and J.A. Bargen, *The Spread of Carcinoma of the Rectum: Invasion of Lymphatics, Veins and Nerves.* Ann Surg, 1943. **118**(1): p. 76-90.
50. Shiozawa, J., et al., *Expression of matrix metalloproteinase-1 in human colorectal carcinoma.* Mod Pathol, 2000. **13**(9): p. 925-33.
51. Shirouzu, K., et al., *Clinicopathologic study of perineural invasion in rectal cancer.* Kurume Med J, 1992. **39**(1): p. 41-9.
52. Silberfein, E.J., et al., *Long-term survival and recurrence outcomes following surgery for distal rectal cancer.* Ann Surg Oncol, 2010. **17**(11): p. 2863-9.
53. Sitzler, P.J., et al., *Lymph node involvement and tumor depth in rectal cancers: an analysis of 805 patients.* Dis Colon Rectum, 1997. **40**(12): p. 1472-6.
54. Stor, Z., et al., *Prognostic value of clinical, pathological and immunohistochemical markers in stage II colon cancer patients.* Acta Chir Iugosl, 2008. **55**(3): p. 39-44.
55. Tanaka, M., et al., *Tumor budding at the invasive margin can predict patients at high risk of recurrence after curative surgery for stage II, T3 colon cancer.* Dis Colon Rectum, 2003. **46**(8): p. 1054-9.
56. Ting, W.C., et al., *Common genetic variants in Wnt signaling pathway genes as potential prognostic biomarkers for colorectal cancer.* PLoS One, 2013. **8**(2): p. e56196.
57. Ueno, H., K. Hase, and H. Mochizuki, *Criteria for extramural perineural invasion as a prognostic factor in rectal cancer.* Br J Surg, 2001. **88**(7): p. 994-1000.
58. Ueno, H., et al., *Characterization of perineural invasion as a component of colorectal cancer staging.* Am J Surg Pathol, 2013. **37**(10): p. 1542-9.
59. Viana Lde, S., et al., *Relationship between the expression of the extracellular matrix genes SPARC, SPP1, FN1, ITGA5 and ITGAV and clinicopathological parameters of tumor progression and colorectal cancer dissemination.* Oncology, 2013. **84**(2): p. 81-91.
60. Weiser, M.R., et al., *Individualized prediction of colon cancer recurrence using a nomogram.* J Clin Oncol, 2008. **26**(3): p. 380-5.
61. Wied, U., et al., *Postoperative survival of patients with potentially curable cancer of the colon.* Dis Colon Rectum, 1985. **28**(5): p. 333-5.
62. Yun, H.R., et al., *The prognostic factors of stage IV colorectal cancer and assessment of proper treatment according to the patient's status.* Int J Colorectal Dis, 2007. **22**(11): p. 1301-10.
63. Matsushima, T., et al., *Preoperative estimation of neural invasion in rectal carcinoma.* Oncol Rep, 1998. **5**(1): p. 73-6.
64. Marchesi, F., et al., *Role of CX3CR1/CX3CL1 axis in primary and secondary involvement of the nervous system by cancer.* J Neuroimmunol, 2010. **224**(1-2): p. 39-44.
65. Fujita, S., et al., *Cancer invasion to Auerbach's plexus is an important prognostic factor in patients with pT3-pT4 colorectal cancer.* Dis Colon Rectum, 2007. **50**(11): p. 1860-6.

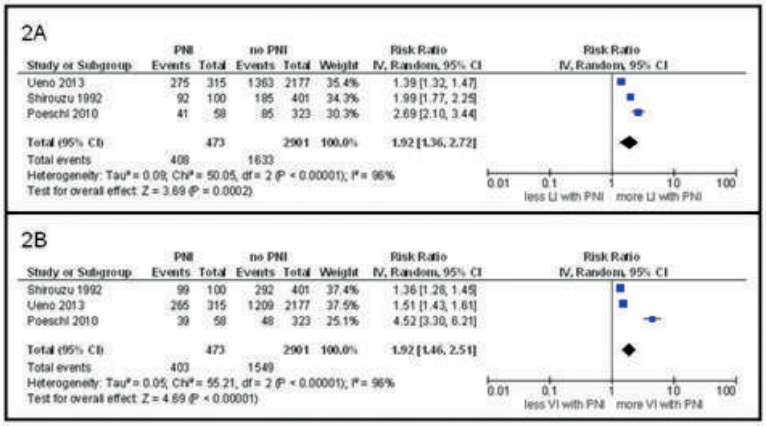
Supplementary table 1

Quality of Reporting	**yes(1)/no(0)/ not applicable (n.a.)**
Specifies criteria for PNI positivity	
Describes the number of slides examined for PNI	
Describes the number of independent blinded scorers of PNI	
Defines LR	
Defines DFS	
Defines OS	
Defines CSS	
Describes the end of follow-up period/date	
Reports the median follow-up time	
Mentions the hospital where the samples come from	
Mentions the time frame of included samples	
Describes sample selection (inclusion/exclusion criteria)	
>90% of initial cases included in UV/MV analysis	
Mentions location (minimally rectum/colon)	
Describes pre-operative treatment details	
Lists freq of patients with T stage; N stage; M stage; perineural invasion	
Reports the relation of PNI to standard prognostic variables	
Reports the estimated effect for PNI on survival in UV analysis (RR, CI and p-value; freq in table)	
Reports the estimated effect (HR, CI and p-value provided) for PNI on survival in MV analysis	
Reports the estimated effects (HR, CI and p-values provided) of all other prognostic factors included in the MV analysis of point 19	
Percentage of reported items	total score/no. of applicable items*100

Supplementary figure 1



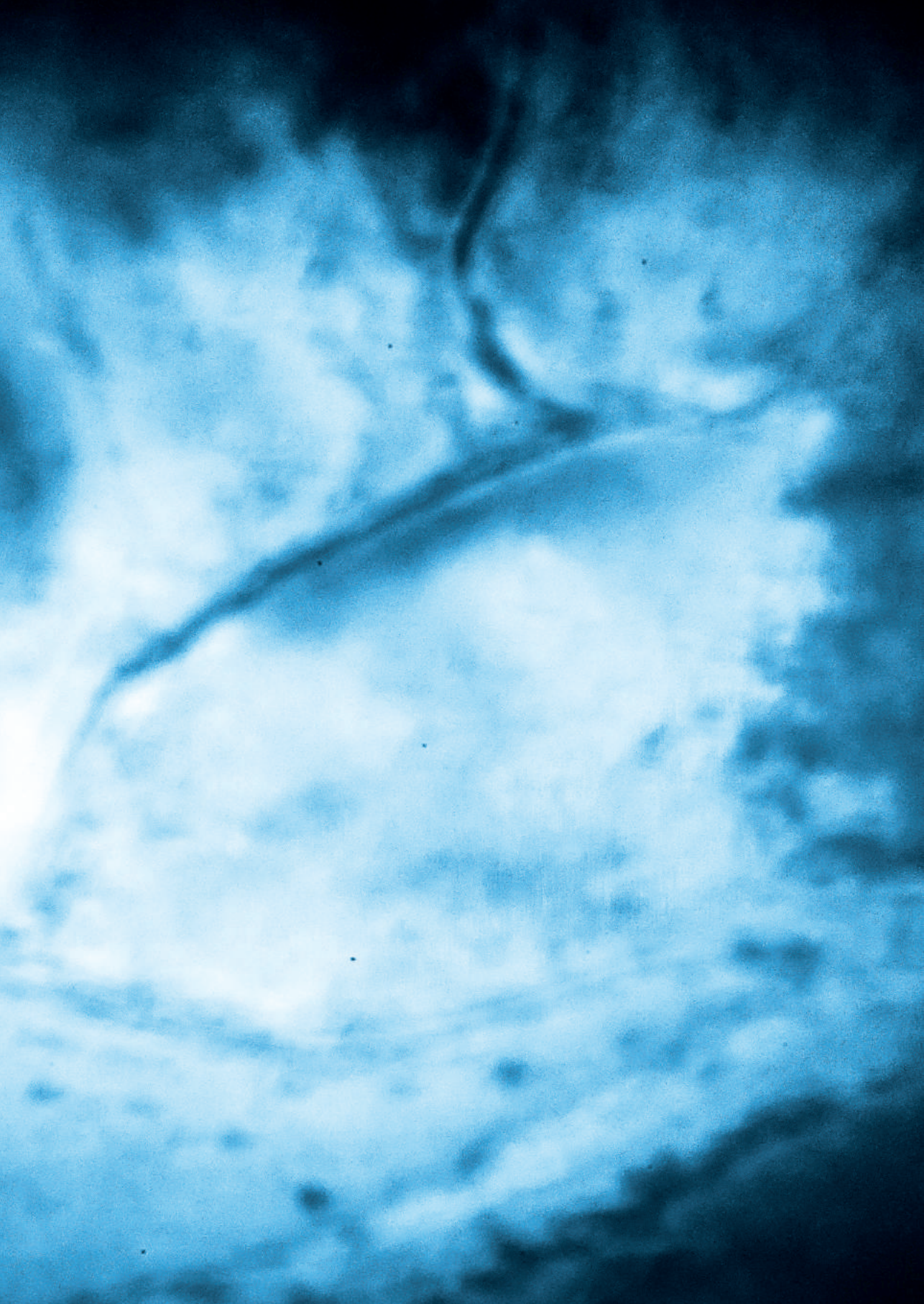
Supplementary figure 2



Supplementary table 2

Quality of Reporting	Allard 2012	Ceyhan 2010	Cohn 1997	Desolneux 2010	Guerra 1997	Horn 1990	Huang 2012	Huh 2013	Jee 2011	Kang 2011	Kim 2011	Knudsen 1983
Specifies criteria for PNI positivity	0	1	0	0	0	0	0	0	0	0	0	0
Describes the number of slides examined for PNI	0	1	0	0	0	1	0	0	0	0	0	1
Describes the number of scorers of PNI	1	1	0	0	0	1	0	0	0	1	0	0
Defines LR	na	0	na	na	na	0	na	1	na	na	1	na
Defines DFS	1	na	na	na	na	na	na	1	0	na	na	na
Defines OS	na	na	0	1	0	0	1	1	0	1	0	1
Defines CSS	na	0	na	na	na	na	1	na	na	na	na	0
Describes the end of follow-up period/date	0	0	0	0	0	1	1	0	0	1	0	1
Reports the median follow-up time	1	1	1	1	0	0	1	1	1	1	1	0
Mentions the hospital of included samples	1	1	1	0	0	0	1	0	1	1	1	1
Mentions the time frame of included samples	1	1	1	1	0	0	1	1	1	1	1	1
Describes sample selection (in/exclusion criteria)	1	1	0	1	1	1	1	1	1	1	1	1
>90% of initial cases included in UV/MV analysis	1	1	1	1	1	1	1	1	1	0	1	1
Mentions location (minimally rectum/colon)	0	1	1	1	0	1	1	1	1	1	1	1
Describes pre-operative treatment details	1	1	0	1	0	1	0	1	na	1	1	0
Lists freq of patients with T,N,M-stage and PNI	1	1	0	1	0	1	1	1	1	0	1	0
Reports relation of PNI to standard prognostic variables	1	1	0	0	0	0	0	0	0	0	0	1
Reports effect for PNI in UV analysis (RR/CI/p-value; freq in table)	0	1	1	1	1	1	1	1	1	1	1	0
Reports effect for PNI in MV analysis (HR/CI/p-value)	0	1	1	1	0	0	1	1	1	1	1	1
Reports effects for all other prognostic variables included in the MV analysis.	0	1	1	0	0	0	1	1	1	0	0	0
Percentage of reported items (=total score/no of applicable items*100)	58.8	83.3	47.1	58.8	17.7	50.0	72.2	68.4	58.8	64.7	61.1	58.8

Law 2009	Lennon 2003	Liebig 2009	Liebl 2013	Lin 2013	Lim 2012	Mulcahy 1993	Oh 2009	Onata Ocana 2004	Pages 2005	Peng 2011	Poeschl 2010	Rullier 2005	Seefeld 1943	Silberfein 2010	Ting 2013	Ueno 2001	Ueno 2013	Yun 2007
0	0	1	1	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0
0	1	1	1	0	0	1	0	1	1	1	1	1	0	0	0	1	0	0
0	1	1	1	0	0	1	0	1	1	1	0	0	1	0	0	1	0	0
1	na	na	0	na	1	na	na	0	0	1	na	1	1	1	na	1	na	na
na	na	1	na	na	1	na	1	na	1	1	0	0	na	na	1	na	0	0
1	na	1	na	1	na	0	1	na	1	0	na	0	0	0	1	0	na	1
na	0	0	0	na	0	na	na	1	na	na	0	na	na	na	na	na	1	na
0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	1	0	0	0
1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
1	0	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0
1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	0	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	0
1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1
1	na	1	na	1	1	1	na	0	0	1	0	1	0	1	0	1	1	0
0	0	1	1	0	1	0	1	1	1	na	1	1	0	0	0	1	1	0
0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	0	1	1	0
1	1	1	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1
1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1
0	0	1	1	1	1	0	0	1	1	0	1	0	0	1	1	0	0	0
61.1	50.0	84.2	76.5	58.8	52.6	64.7	64.7	72.2	73.7	83.3	61.1	47.3	50.0	61.1	61.1	83.3	72.2	38.9



Chapter 8

The value of intramural vascular invasion in colorectal cancer – a systematic review and meta-analysis

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Abstract

Extramural venous invasion (EMVI) is a well-known prognostic factor in colorectal cancer (CRC). Vascular invasion within the bowel wall, intramural vascular invasion (IMVI), has received less attention and its incidence and prognostic importance in CRC is not completely known.

A systematic literature search was performed focusing on the impact of IMVI in CRC. Data were analysed using Review Manager 5.3 on incidence and clinical endpoints local recurrence, five year cancer specific survival (CSS) and five year overall survival (OS). Meta-analysis was performed in terms of risk ratios (RR) and hazard ratios (HR) with 95% confidence interval (95%CI).

Of the initial 1199 articles identified by our search strategy, 20 were included in this meta-analysis. Of the 8078 included patients, 1008 patients had IMVI (12.5%). Studies that re-examined histological slides showed a higher incidence of IMVI compared to studies extracting IMVI from pathology reports (17.6% vs. 7.7%, $p<0.001$). Detection of IMVI increased significantly with the use of additional staining (22.9% vs. 12.3%, $p<0.001$). IMVI was associated with a decreased CSS (HR 1.6, 95%CI 1.2-2.2 in multivariate analysis). A borderline significant effect was observed for IMVI on local recurrence (RR 1.5, 95%CI 0.98-2.3) and OS (RR 1.2, 95%CI 1.0-1.4).

In conclusion, despite the limited number of studies, there is a clear association with outcome in the presence of IMVI. This warrants more attention to this underreported prognostic factor.

Introduction

The presence of tumour cells in veins outside the bowel wall, extramural venous invasion (EMVI), is a well-known prognostic factor in colorectal cancer (CRC). In fact, in stage II patients, EMVI is one of the indications for adjuvant chemotherapy.[1] Its strong association with the development of particularly liver metastases[2] seems to justify this decision. EMVI is therefore a mandatory item in many guidelines and datasets for reporting CRC. The Royal College of Pathologists has set a standard detection rate for EMVI at 25% and a minimum of four tumour blocks per specimen is advised for optimal determination of its presence.[3] Vascular invasion *within* the bowel wall, intramural vascular invasion (IMVI), has received less attention and its prognostic importance in CRC is not entirely clear.

There is a wide variation in reported frequencies of IMVI in CRC, ranging from 8% to 39%.[4, 5] Analogous to EMVI, this variation might be explained by differences in pathological assessment.[6, 7] The incidence of vascular invasion increases with the number of tumour blocks analysed and with the use of additional elastic stains.[8-10]

Some studies demonstrated an association between IMVI and development of distant metastases,[10, 11] suggesting that the *presence* of venous invasion may be more important than its *location* in the bowel wall.

To investigate the impact of IMVI in CRC, we systematically reviewed the frequency and impact of IMVI in CRC. The prognostic endpoints are local recurrence, five year overall survival (5yOS) and five year cancer specific survival (5yCSS).

Materials and methods

Search strategy and selection

A comprehensive literature search was performed using the PubMed database from inception to 1 January 2016, using the following keywords: "Colorectal Neoplasms"[Mesh] or "colorectal"[Title/Abstract] or "colon"[Title/Abstract] or "rectum"[Title/Abstract] or "rectal"[Title/Abstract] and "cancer"[Title/Abstract] or "carcinomas"[Title/Abstract] or "neoplasms"[Title/Abstract] or "tumors"[Title/Abstract] and "vascular"[Text Word] or "venous"[Text Word] or "veins"[Text Word] or "vessels"[Text Word] and "invasion"[Text Word] or "spread"[Text Word] and "prognosis"[Text Word] or "prognostic"[Text Word] or "survival"[Text Word] or "predict"[Text Word]. Additional searches were performed by manual cross-referencing.

Two independent investigators (NK,UvE) reviewed each report for eligibility. Only articles that were published in English were selected. Studies with and without neoadjuvant treatment were included. In case of overlapping patient data, as a

consequence of subsequent publication of updated series, results of the largest study or the study with longest follow-up were included in this meta-analysis.

Data extraction

Data on tumour stage, histologic factors, method of IMVI detection, incidence of IMVI and EMVI were extracted. Data on prognostic factors, local recurrence, 5yOS and 5yCSS from univariate and multivariate analyses were collected and entered in Review Manager Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012. Analyses were performed with a minimum of two studies for each endpoint. In case multiple studies used different point estimates (e.g. risk ratio (RR) or hazard ratio (HR)) on the same survival endpoint, the point estimate used in the largest number of articles was included in our meta-analysis.

Quality assessment and risk of bias

A scale to assess the quality of the reporting of the included articles was developed, based on the REMARK guidelines, specifically looking at reporting of IMVI.[12, 13] Only studies with data on outcome were subjected to quality assessment. Scoring was performed by two independent investigators (NK,UvE). In case of disagreement, a consensus score was agreed after discussion. The association between quality of reporting and the RR/HR was analysed with scatter plots and non-parametric correlation testing. Publication bias was assessed through visual inspection of symmetry of funnel plots.

Statistical analysis

A meta-analysis was performed with all available studies on each endpoint, in terms of RR/HR with 95%CI. If no HR was reported, it was calculated from Kaplan-Meier curves.[14] Univariate and multivariate analyses were subdivided. A random effects model with inverse variance weighting of studies was used. Heterogeneity was assessed using a χ^2 test, with a p-value of <0.10 to show the presence of significant heterogeneity. In case of heterogeneity, sub-analyses were performed to identify the potential source of heterogeneity.

Results

Search results

A total of 1199 potentially relevant articles were retrieved by the database search. 1181 articles were excluded because they did not meet inclusion criteria (Figure 1). Manual cross-referencing resulted in two additional articles. The remaining 20 articles, comprising 8078 patients, were included in the meta-analysis.[4, 5, 8, 9, 11, 15-28]

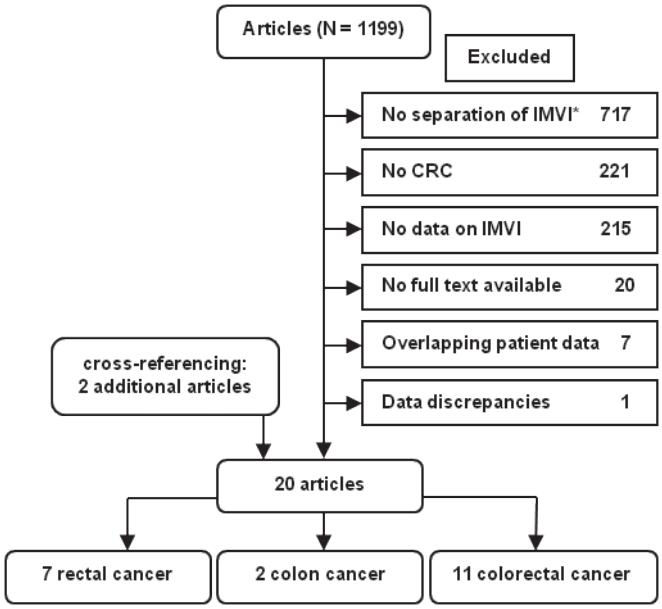


Figure 1: Flow chart of search strategy. Abbreviations: IMVI: intramural vascular invasion, CRC: colorectal carcinoma, *lymphatic and vascular invasion were grouped together or vascular invasion was not subdivided into intramural or extramural vascular invasion.

Seven studies investigated IMVI in rectal cancer (2342 patients), two in colon cancer (562 patients) and in 11 rectal and colon carcinomas were grouped together (5174 patients). The main characteristics are shown in table 1. The mean number of patients per study included in our meta-analysis was 404 (range 34-3040).

Quality of reporting of the included articles

Studies with outcome data (n=9) were subjected to quality assessment, focused on the quality of reporting of IMVI.[4, 11, 15, 17, 18, 24-27] The mean percentage of reported relevant parameters of the included studies was 63.5% (range 38.9-88.9%). Two studies reported less than 50% of required items, four studies reported 50-75% of required items and three studies reported >75% of items.

Frequency of IMVI and association with other prognostic factors

The overall published incidence of IMVI was 12.5% (table 1). The percentage of IMVI was significantly higher in studies that re-examined pathologic slides (16 studies; 3899 patients) versus studies that extracted IMVI from pathology reports (4 studies; 4179 patients) (17.6% vs. 7.7%, p<0.001). In the studies that re-examined pathologic slides, the use of additional staining (9 studies; 1959 patients) increased detection even more compared to routine Haematoxilyn and Eosin (HE) (7 studies; 1940 patients) (22.9% vs. 12.3%, p<0.001). Only one article[15] directly compared HE- and Elastina von Gieson (EvG) staining: IMVI was detected in 3.2% of the HE stained slides and in 14.1% of the EvG stained slides. The frequency of IMVI also increased with TNM stage. A low incidence was detected in patients with stage I disease (6.0%, total 684 cases). Higher incidences were found in CRC stage II, III and IV (11.6%, total cases 2182, 14.3%, total cases 1339 and 19.6%, total cases 689, respectively, (p<0.001)). 19 studies (7653 patients) provided data on both IMVI and EMVI, the overall incidence of EMVI was 24.3% in those studies, compared to 11.7% of IMVI. Eight of these publications (4151 patients)[4, 5, 8, 10, 11, 16, 21, 22] subdivided the incidence of vascular invasion in three groups; IMVI-only, EMVI-only, both IMVI and EMVI. IMVI-only was detected in 7.1%, EMVI-only in 15.3%, both IMVI and EMVI in 5.1% of patients.

Effect of IMVI on local recurrence

Data on the impact of IMVI on local recurrence in univariable analysis could be extracted from two articles, which included 503 patients.[17, 25] The presence of IMVI was not significantly associated with local recurrence (RR 1.5, 95%CI 0.98-2.3, p=0.06) (Figure 2A). No heterogeneity was observed (χ^2=0.46, df=1 (p=0.50); I^2=0%). Both studies showed comparable RR and no heterogeneity, therefore subgroup analysis was not performed. Dresen et al.17 provided data on multivariate analysis, confirming the lack of association between IMVI and local recurrence (OR 1.9, 95%CI 0.7-5.1).

Effect of IMVI on overall survival

Data on the impact of IMVI on 5yOS in univariable analysis could be extracted from five studies, which included 2117 patients.[4, 15, 18, 25, 26] The 5yOS decreased in the presence of IMVI (RR 1.2, 95%CI 1.0-1.4, p=0.02) (Figure 2B). Some heterogeneity was observed (χ^2=11.87, df=6 (p=0.07); I^2=49%), which could not be explained by differences in sample size, timeframe and TNM stage. Despite the observed heterogeneity, the direction of the effect in the forest plots was rather consistent. There was no asymmetry in funnel plot. The percentage of items reported in the individual studies varied from 38.9% to 84.2%, and did not correlate with the magnitude of RR (Spearman r=-0.60, p=0.35). The studies in which IMVI was extracted from pathology reports[4, 18] showed

similar RR compared to those with specific review for IMVI[15, 25, 26] (RR 1.3, 95%CI 0.98-1.7 and RR 1.2, 95%CI 0.98-1.4, respectively).

Table 1: Main characteristics of 20 included studies with data on IMVI.

Author	Location	Stage	Method of IMVI detection	Staining used	No. observers	Used for outcome	No. pts	% IMVI
Baumhoer[15]	Colorectal	I-II	Revision	HE, Elastic stain	nm	5yOS UV	185	14.1%
Betge[11]	Colorectal	I-IV	Revision	HE	2	5yCSS UV+MV	381	8.7%
Cavdar[16]	Rectum	I-IV	Extraction from PA reports	-	-	-	34	14.7%
Dresen[17]	Rectum	I-III	Revision	HE	2	LR UV+MV	277	10.1%
Freedman[18]	Rectum	I-III	Extraction from PA reports	-	-	5yOS UV	673	6.7%
Gibson[4]	Colorectal	I-IV	Extraction from PA reports	-	-	5yOS UV+MV, 5yCSS UV+MV	3040	7.5%
Hayes[19]	Colorectal	I-III	Revision	HE	1	-	179	14.0%
Howlett[8]	Colorectal	I-IV	Revision	HE, Elastic stain	3	-	92	27.2%
Inoue[9]	Colorectal	I-III	Revision	HE, Elastic stain	2	-	94	20.2%
Krasna[20]	Colorectal	I-III	Revision	HE	2	-	77	3.9%
Minsky[21]	Colon	I-III	Revision	HE, Elastic stain	1	-	294	32.3%
Minsky[5]	Rectum	I-III	Revision	HE, Elastic stain	1	-	168	38.7%
Ouchi[22]	Colorectal	III-IV	Revision	HE, Elastic stain	nm	-	61	29.5%
Petersen[28]	Colon	II	Revision	HE	1	-	268	9.0%
Prabhudesai[23]	Rectum	I-IV	Revision	HE	2	-	55	25.5%
Roxburgh[24]	Colorectal	I-III	Revision	HE, Elastic stain	2	5yCSS UV	559	10.6%
Shirouzu[25]	Colorectal	II-III	Revision	HE, Elastic stain	1	5yOS UV, LR UV	425	25.9%
Sternberg[10]	Colorectal	IV	Revision	HE, Elastic stain	2	-	81	38.3%
Talbot[26]	Rectum	II	Revision	HE	nm	5yOS UV	703	15.8%
Tilney[27]	Rectum	I-III	Extraction from PA reports	-	-	5yCSS UV+MV	432	10.0%

Abbreviations: IMVI: intramural vascular invasion; no: number of; pts: patients; nm: not mentioned; HE: Haematoxylin and Eosin; LR: local recurrence; 5yCSS: 5 year cancer specific survival; 5yOS: 5 year overall survival; UV: univariate; MV: multivariate.

Two articles analysed stage-dependent 5yOS.[4, 25] Shirouzu et al.[25] found no effect of IMVI in stage II, nor in stage III patients. In contrast, Gibson et al.[4] found a significant effect of IMVI on 5yOS in stage III and IV patients, but not in stage II patients.

Gibson et al.[4] provided data on multivariate analysis, showing IMVI to be an independent prognostic factor in stage III CRC (HR 1.5, 95%CI 1.1-2.2, p=0.02). Other independent prognostic factors reported in stage III CRC were EMVI (HR 1.5, 95%CI 1.2-1.8), age≥75 (HR 2.0, 95%CI 1.6-2.4), male sex (HR 1.3, 95%CI 1.1-1.6), T3 tumours (HR 1.3, 95%CI 1.1-1.7), T4 tumours (combined HR 1.6, 95%CI 1.2-2.1), apical node involvement (HR 1.7, 95%CI 1.3-2.3), ≥30% of nodes involved (combined HR 1.5, 95%CI 1.3-1.8), high grade (HR 1.3, 95%CI 1.1-1.6), postoperative chemotherapy (HR 0.7, 95%CI 0.5-0.9) and year of resection (HR 0.98. 95%CI 0.96-0.99). In stage IV CRC, IMVI was not independently associated with OS (HR 1.4, 95%CI 0.9-2.3, p=0.14).

Effect of IMVI on cancer specific survival

The impact of IMVI on 5yCSS in univariate analysis could be extracted from four articles, which included 1620 patients.[4, 11, 24, 27] The presence of IMVI is associated with worse 5yCSS (HR 2.0, 95%CI 1.4-3.0, p<0.001) (Figure 2C). Some heterogeneity was observed (χ^2=6.69, df=3 (p=0.08); I^2=55%), which could not be explained by differences in sample size, timeframe and TNM stage. Despite the observed heterogeneity, the direction of the effect in the forest plots was rather consistent. There was no asymmetry in funnel plot. With respect to quality of the included publications, percentage of items reported ranged from 57.9% to 88.9%. This did not correlate with the magnitude of HR (Spearman r=-0.21, p=0.92). Articles in which IMVI was extracted from pathology reports[4, 27] showed a lower HR compared to studies with specific review for IMVI[11, 24] (HR 1.6, 95%CI 1.2-2.0 and HR 3.3, 95%CI 2.0-5.5, respectively).

Multivariate analysis was performed in three studies comprising 1283 patients, confirming a decreased 5yCSS in the presence of IMVI (HR 1.6, 95%CI 1.2-2.2, p<0.001) (Figure 2D).[4, 11, 27] No heterogeneity was observed (χ^2=0.16, df=2 (p=0.92); I^2=0%), there was no asymmetry in funnel plot. The percentage of items reported varied from 57.9% to 88.9% and this did not correlate with the magnitude of HR (Spearman r=0.50, p=1.00). Articles in which IMVI was extracted from pathology reports[4, 27] showed similar HR compared to the study with specific review for IMVI[11] (HR 1.6, 95%CI 1.1-2.3 and HR 1.8, 95%CI 0.7-4.2, respectively).

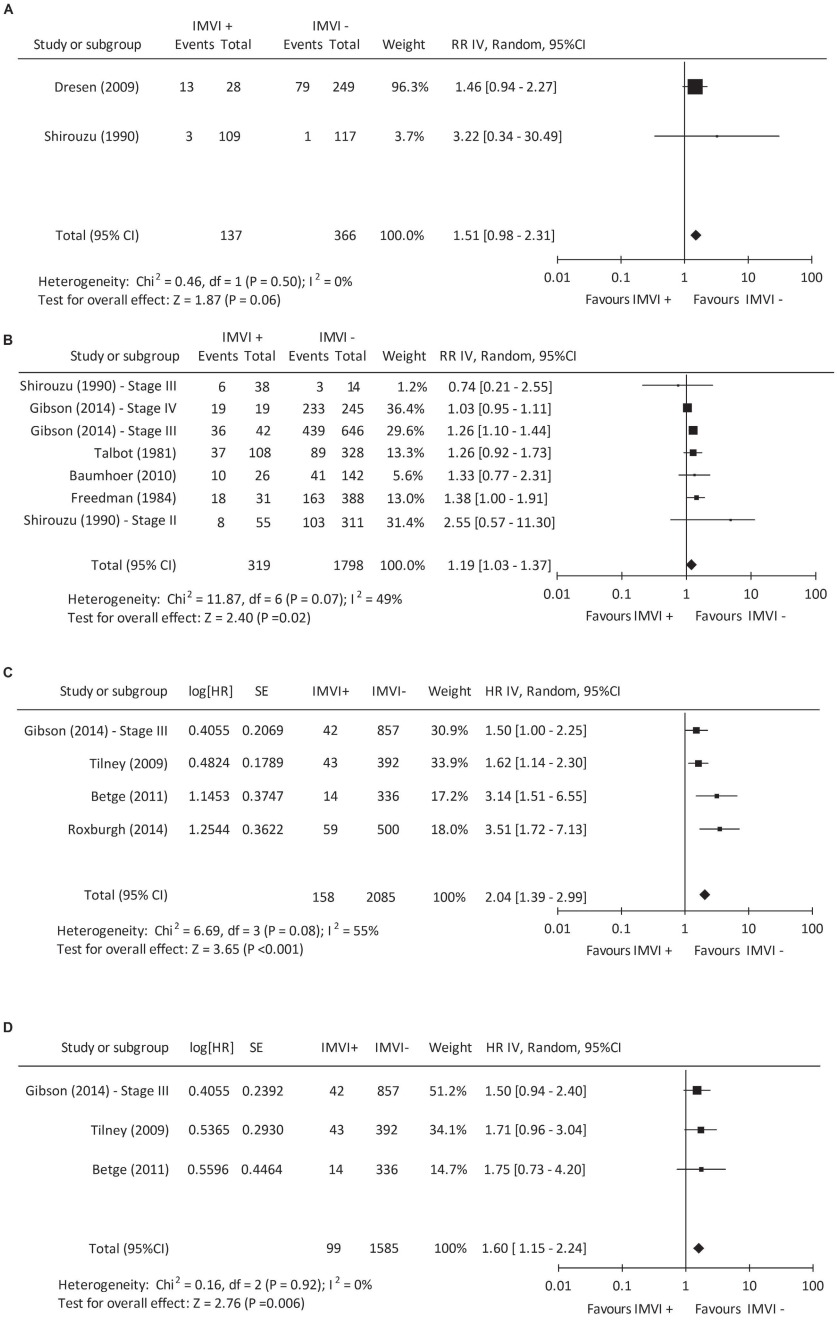


Figure 2: The impact of IMVI on outcome in univariate and multivariate analysis. A) Impact of IMVI on LR, UV analysis. B) Impact of IMVI on 5yOS, UV analysis. C) Impact of IMVI on 5yCSS, UV analysis. D) Impact of IMVI on 5yCSS, MV analysis. Abbreviations: IMVI: intramural vascular invasion; IV: inverse variance; RR: risk ratio; HR: hazard ratio; CI: confidence interval; LR: local recurrence; 5yCSS: 5 year cancer specific survival; 5yOS: 5 year overall survival.

Other independent prognostic factors for 5yCSS in multivariate analysis reported in all three studies were T-stage and EMVI. T-stage T3/T4 showed a combined HR of 3.2 (95%CI 1.4-7.3). EMVI showed a combined HR of 1.7 (95%CI 1.4-2.1). Tumour grade was reported in two studies and provided a combined HR of 1.5 (95%CI 1.2-1.8).[4, 11]

Comparison with EMVI

Six articles[4, 11, 15, 24, 25, 27] included in our meta-analysis compared the effect of IMVI with EMVI on survival. Four studies[4, 11, 24, 27] reported on the impact of EMVI on 5yCSS in univariate analysis, showing worse survival in the presence of EMVI (HR 3.6, 95%CI 2.4-5.5). Three studies[4, 11, 27] provided data on the impact of EMVI on 5yCSS in multivariate analysis, showing worse outcome (HR 1.7, 95%CI 1.4-2.1). These HR are not statistically different from the HR of IMVI in the same studies (univariate analysis HR 2.0, 95%CI 1.4-3.0, multivariate analysis HR 1.6, 95%CI 1.1-2.2). Baumhoer et al.[15] also detected no significant differences in 5yOS of patients with IMVI or EMVI (62% vs. 74%, p=0.473). Shirouzu et al.[25] analysed three different subcategories of vascular invasion; IMVI-only, IMVI more prominent than EMVI and EMVI more prominent than IMVI. In stage II CRC the survival rates were not significantly different for IMVI-only, IMVI more prominent than EMVI and EMVI more prominent than IMVI (85.8%, 87.3% and 82.9%, respectively). In stage III CRC survival of patients with EMVI more prominent than IMVI was significantly worse compared to patients with IMVI-only and IMVI more prominent than EMVI (45% vs. 84.9% and 73.3%, $p<0.005$ and $p<0.001$, respectively).

Discussion

In the current meta-analysis we have shown that IMVI, with an overall published incidence of 12.5%, is a prognostic factor in CRC. Despite its prognostic importance, IMVI is currently underreported: specific revision aimed on the detection of IMVI showed an increase of 10% compared to standard diagnostic evaluation in published studies.

Ideally, potential new histological markers should be compared to established biomarkers to interpret their additional value. Unfortunately, in this meta-analysis it was not possible to compare IMVI to other risk factors in CRC. Each individual study included different prognostic factors, such as age, lymph node metastases, tumour location or circumferential resection margin in their multivariate analysis.[4, 11, 27] Only EMVI and T-stage were included in multiple studies, showing similar HR for EMVI and a higher HR for T-stage, compared to IMVI. It would be interesting to compare potential histological risk factors to other well-known risk factors in CRC, but therefore uniform

definitions are needed. Furthermore, ideally all risk factors should be included in outcome analysis per study.

Differences in studies reporting oncologic outcomes hampered our investigation. In some studies local and distant recurrences were reported together, and different outcome measurements were used, decreasing the number of studies that could be analysed. Due to the limited number of studies included in this meta-analysis, interesting subgroup analyses could not be performed. For example, it would be good to know whether the increased detection of IMVI due to additional elastic staining also results in an improved prognostic power. In the literature, there are many studies that were potentially interesting, but did not report IMVI separately from either EMVI or lymphatic invasion. This was the main point of exclusion in our current study, accounting for 717 exclusions.

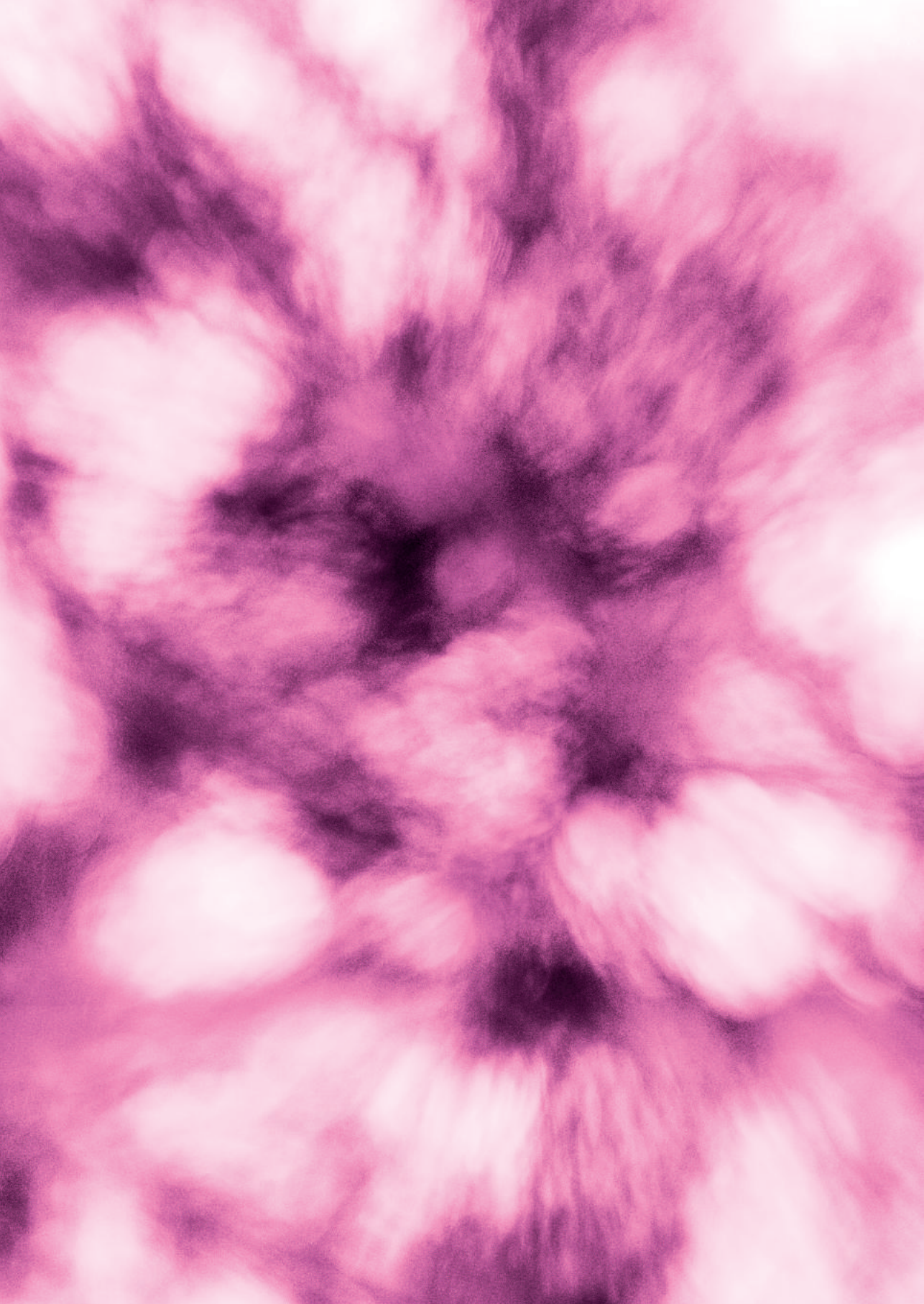
The articles included in our meta-analysis showed a detection rate of EMVI of 24.3%, nearly twice the detection rate of IMVI. A comparable percentage of EMVI prevalence was found in a recent meta-analysis (26%).[29] Six articles in our meta-analysis directly compared the impact of IMVI with EMVI and at least four studies showed no significant difference in prognostic impact. This supports the AJCC/UICC TNM classification and the current Japanese guidelines, where IMVI and EMVI are not separated.[30, 31] However, a large study[32] including 2405 patients, showed a HR of 2.8, (95%CI 2.1-3.8) on overall survival in multivariate analysis, which seems higher than the HR of IMVI found in our meta-analysis. A recent meta-analysis[29] also linked EMVI to worse oncological outcome in rectal cancer with a pooled overall survival of 39.5% (95%CI 0.29-0.51). In that particular meta-analysis all studies were published between 1943 and 1988, which explains the poor survival outcome and limits its current relevance.

In conclusion, despite the limited number of studies, our meta-analysis clearly shows decreased outcomes in the presence of IMVI. A direct comparison of IMVI with EMVI showed no significant difference in prognostic impact, supporting the idea that the *presence* of vascular invasion is more important than its exact *location*. Since its relevance to better clinical outcome, more attention to this underreported prognostic factor seems justified.

References

1. Figueredo A, Coombes ME, Mukherjee S. Adjuvant therapy for completely resected stage ii colon cancer. *The Cochrane database of systematic reviews* 2008;CD005390.
2. Nagtegaal ID, Knijn N, Hugen N *et al.* Tumor deposits in colorectal cancer: Improving the value of modern staging-a systematic review and meta-analysis. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2017;**35**;1119-1127.
3. http://www.rcpath.org/Resources/RCPath/Migrated%20Resources/Documents/G/G049_ColorectalDataset_July14.pdf, 2014
4. Gibson KM, Chan C, Chapuis PH, Dent OF, Bokey L. Mural and extramural venous invasion and prognosis in colorectal cancer. *Diseases of the colon and rectum* 2014;**57**;916-926.
5. Minsky BD, Mies C, Recht A, Rich TA, Chaffey JT. Resectable adenocarcinoma of the rectosigmoid and rectum. Ii. The influence of blood vessel invasion. *Cancer* 1988;**61**;1417-1424.
6. Maguire A, Sheahan K. Controversies in the pathological assessment of colorectal cancer. *World journal of gastroenterology : WJG* 2014;**20**;9850-9861.
7. Messenger DE, Driman DK, Kirsch R. Developments in the assessment of venous invasion in colorectal cancer: Implications for future practice and patient outcome. *Human pathology* 2012;**43**;965-973.
8. Howlett CJ, Tweedie EJ, Driman DK. Use of an elastic stain to show venous invasion in colorectal carcinoma: A simple technique for detection of an important prognostic factor. *Journal of clinical pathology* 2009;**62**;1021-1025.
9. Inoue T, Mori M, Shimono R, Kuwano H, Sugimachi K. Vascular invasion of colorectal carcinoma readily visible with certain stains. *Diseases of the colon and rectum* 1992;**35**;34-39.
10. Sternberg A, Amar M, Alfici R, Groisman G. Conclusions from a study of venous invasion in stage iv colorectal adenocarcinoma. *Journal of clinical pathology* 2002;**55**;17-21.
11. Betge J, Pollheimer MJ, Lindtner RA *et al.* Intramural and extramural vascular invasion in colorectal cancer: Prognostic significance and quality of pathology reporting. *Cancer* 2012;**118**;628-638.
12. Knijn N, Simmer F, Nagtegaal ID. Recommendations for reporting histopathology studies: A proposal. *Virchows Archiv : an international journal of pathology* 2015.
13. McShane LM, Altman DG, Sauerbrei W *et al.* Reporting recommendations for tumor marker prognostic studies. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2005;**23**;9067-9072.
14. Parmar MK, Torri V, Stewart L. Extracting summary statistics to perform meta-analyses of the published literature for survival endpoints. *Stat Med* 1998;**17**;2815-2834.
15. Baumhoer D, Thiesler T, Maurer CA, Huber A, Cathomas G. Impact of using elastic stains for detection of venous invasion in the prognosis of patients with lymph node negative colorectal cancer. *International journal of colorectal disease* 2010;**25**;741-746.
16. Cavdar Z, Canda AE, Terzi C, Sarioglu S, Fuzun M, Oktay G. Role of gelatinases (matrix metalloproteinases 2 and 9), vascular endothelial growth factor and endostatin on clinicopathological behaviour of rectal cancer. *Colorectal disease : the official journal of the Association of Coloproctology of Great Britain and Ireland* 2011;**13**;154-160.
17. Dresen RC, Peters EE, Rutten HJ *et al.* Local recurrence in rectal cancer can be predicted by histopathological factors. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology* 2009;**35**;1071-1077.
18. Freedman LS, Macaskill P, Smith AN. Multivariate analysis of prognostic factors for operable rectal cancer. *Lancet* 1984;**2**;733-736.
19. Hayes BD, O'Riordan JM, Stuart C, Muldoon C. Rectal site and suboptimal nodal yield predict systemic recurrence in resected colorectal carcinoma: A case-control study. *International journal of surgical pathology* 2014;**22**;505-511.

20. Krasna MJ, Flancbaum L, Cody RP, Shneibaum S, Ben Ari G. Vascular and neural invasion in colorectal carcinoma. Incidence and prognostic significance. *Cancer* 1988;**61**;1018-1023.
21. Minsky BD, Mies C, Rich TA, Recht A, Chaffey JT. Potentially curative surgery of colon cancer: The influence of blood vessel invasion. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 1988;**6**;119-127.
22. Ouchi K, Sugawara T, Ono H *et al.* Histologic features and clinical significance of venous invasion in colorectal carcinoma with hepatic metastasis. *Cancer* 1996;**78**;2313-2317.
23. Prabhudesai A, Arif S, Finlayson CJ, Kumar D. Impact of microscopic extranodal tumor deposits on the outcome of patients with rectal cancer. *Diseases of the colon and rectum* 2003;**46**;1531-1537.
24. Roxburgh CS, McMillan DC, Richards CH *et al.* The clinical utility of the combination of t stage and venous invasion to predict survival in patients undergoing surgery for colorectal cancer. *Annals of surgery* 2014;**259**;1156-1165.
25. Shirouzu K, Isomoto H, Kakegawa T, Morimatsu M. A prospective clinicopathologic study of venous invasion in colorectal cancer. *American journal of surgery* 1991;**162**;216-222.
26. Talbot IC, Ritchie S, Leighton M, Hughes AO, Bussey HJ, Morson BC. Invasion of veins by carcinoma of rectum: Method of detection, histological features and significance. *Histopathology* 1981;**5**;141-163.
27. Tilney HS, Rasheed S, Northover JM, Tekkis PP. The influence of circumferential resection margins on long-term outcomes following rectal cancer surgery. *Diseases of the colon and rectum* 2009;**52**;1723-1729.
28. Petersen VC, Baxter KJ, Love SB, Shepherd NA. Identification of objective pathological prognostic determinants and models of prognosis in dukes' b colon cancer. *Gut* 2002;**51**;65-69.
29. Chand M, Siddiqui MR, Swift I, Brown G. Systematic review of prognostic importance of extramural venous invasion in rectal cancer. *World journal of gastroenterology : WJG* 2016;**22**;1721-1726.
30. *Japanese society for cancer of the colon and rectum: Japanese classification of colorectal carcinoma*. Tokyo, Japan: Kanehara, 2009.
31. Sobin LH, Gospodarowicz MK, Wittekind C. *Tnm classification of malignant tumours*. 7th ed. ed. Oxford: Wiley-Blackwell, 2010.
32. McClelland D, Murray GI. A comprehensive study of extramural venous invasion in colorectal cancer. *PloS one* 2015;**10**;e0144987.



Appendix

Recommendations for reporting histopathology studies: a proposal

A

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Background

Most published histopathology studies (describing histological characteristics of existing or new entities, existing or new markers detected by immunohistochemistry, *in situ* hybridization or molecular methods in tumor material often in relation to patient outcome) are retrospective and use tissue samples from a single center only. This limits the quality of the evidence provided in such a paper. A higher level of evidence, such as would be required to justify implementation in daily clinical practice, can be reached for tissue-based biomarkers by systematic review of published studies and meta-analysis of the provided data.

In such meta-analyses only research data of sufficient quality should be used. Universally accepted criteria for the assessment of data quality do not exist. However, an essential element would be reporting at a sufficient level of detail of the key components that make up the body of evidence presented in any particular paper. This would also facilitate repetition of the experiments performed and of the relevant observations, an essential step as reproducibility is an absolute prerequisite for validation of tissue biomarkers prior to their implementation in clinical practice.

For *in situ* hybridization and immunohistochemistry biomarkers the Minimum Information Specification for in situ hybridization and immunohistochemistry experiments (MISFISHIE) guidelines have been developed to ensure that a report contains sufficient detail of the assay used.[1] MISFISHIE guidelines identify six types of information to be provided for each experiment: experimental design, biomaterials (biospecimens used) and treatments (preanalytical conditions such as fixation and embedding), reporters (antibodies and probes), staining (fluorescence or chromogenic), imaging data (how images were obtained) and image characterization (how information was extracted from the images, including quantification of relevant image elements). However, they do not focus on statistics (correlation of image derived information with clinical data) or interpretation of the results, which are essential elements of a scientific paper.

To improve possibilities to compare results across studies involving molecular prognostic biomarkers, the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines have been developed. These are intended to facilitate evaluation of the appropriateness and quality of study design, used methods, approaches applied to data analysis and presentation of the results.[2] The REMARK guidelines can also be used for reporting of biomarker studies that are not strictly molecular, such as those reporting retrospective histopathological observations, although some items on the checklist will then be less applicable. Notably, building of prognostic models, checking model assumptions, model validation and internal validation might not be feasible.

In view of a perceived need for better standardization of retrospective histopathology studies, we have used the REMARK guidelines as a blueprint for the development of basic rules for their reporting.[3] In analogy with REMARK guidelines, we propose a checklist of 20 items, grouped according to the generally used headings in a research paper: Introduction, Material and Methods, Results, and Discussion. We have put these together in a table and will discuss each of them briefly. The intention of our commentary is to increase awareness of the need for more standardization and to stimulate discussion, in order to get to a generally accepted approach to standardized reporting of histopathological studies (table 1).

Table: Proposed items for reporting histopathology studies

Introduction
States the FOI, the study objectives and hypotheses
Material and Methods
Describes patient characteristics, inclusion and exclusion criteria
Describes (neoadjuvant) treatment details
Describes type of material used and number of slides examined
Specifies criteria for the FOI
Describes the number of independent (blinded) scorers
States the method of case selection, study design, hospital and time period
Describes the end of follow-up period and median follow up time
Defines all clinical end points examined
Specifies all statistical methods
Describes how associations with other clinical/pathological factors were analyzed
Results
Describes the number of patients included in the analysis and reason for drop-out
Reports patient/tumor characteristics (including FOI) with number of missing values
Describes the relation of the FOI to standard prognostic variables
>90% of initial cases included in UV/MV analysis
Reports the estimated effect (RR/OR, CI and p-value provided) in UV analysis
Reports the estimated effect (HR, CI and p-value provided) in MV analysis
Reports the estimated effects (HR, CI and p-values provided) of other prognostic factors included in MV analysis
Discussion
Interprets the results in context of the prespecified hypotheses and other relevant studies; include a discussion of limitations of the study.
Discusses implications for future research and clinical value

Legend: FOI: factor of interest, RR: relative risk, OR: odds ratio, CI: confidence interval, HR: hazard ratio; UV: Univariate; MV: multivariate

The checklist

1. State the marker of interest, study objectives and working hypotheses.
In order to understand the rationale (why this particular marker) and potential clinical applications (what is needed for this particular condition) a description of the marker of interest, study objectives and a working hypotheses are necessary. Describe what is known on the biology of the marker, methods to detect and quantify the marker and why the marker might be of clinical interest. A working hypothesis should be formulated as a rule in terms that can be tested statistically.

2. Describe patient characteristics, inclusion and exclusion criteria.
Describe the clinical context of the study. Describe why a particular cohort of patients was selected and the criteria used to define the cohort, which includes inclusion and exclusion criteria. Describe clinical details of the cohort in relation to potential use of the marker of interest. As an example, when the working hypothesis is that a marker might have a different prognostic value in different stages of disease, disease stage is an essential element in the description of patient data.

3. Describe treatment details.
Treatment (neoadjuvant, adjuvant, first line, second line etc.) is intended to alter the disease course of a patient. Different treatment modalities might not be distributed equally between groups with or without the biomarker, and this will become an important confounding factor when correlation between outcome and marker expression is looked for. Moreover treatment might also have an influence on marker expression if the patient was treated prior to the moment the sample was taken, which will be a confounding factor in the analysis of the impact of the biomarker. When treatment information is missing this should be specifically stated and in studies on marker expression in relation to treatment response such patients should be excluded.

4. Describe type of material used.
Tissue samples used in retrospective studies are often convenience collections, which potentially run a serious risk of collection bias.[4] Authors should report why and how the specimens were collected and how the specimen was handled (primary tumor site or metastatic lesion, biopsy or resection formalin fixed paraffin-embedded or frozen tumor tissue). Where possible, data on pre-analytical handling of specimens should also be given, in order to clarify potential confounding effects associated with sample condition.[5] When control samples are used, their origin should be stated as well as how they were selected. Control samples should fit into the experimental design based upon the working hypothesis, to avoid problems of

unexpected differences between control and patient samples. Authors should report methodological variables as much as possible according to MISFISHIE guidelines[1]).

5. Specify how expression of the biomarker was assessed.

A detailed description of the criteria for assessment of the presence or absence of the biomarker at tissue level allows evaluation of potential shortcomings but also will enable future researchers to reproduce the study. Some retrospective studies on classical pathological markers tend to extract data from pathology reports, instead of rereading the slides or repeating marker expression analysis for the purpose of the investigation. This runs a risk of heterogeneity between method runs or methods applied and problems of lack of inter-individual reproducibility in reading the results. This can lead to over- or underestimation of the number of patients expressing a certain marker and might introduce selection bias.[6] For purely morphological (gross or microscopical) markers details of specimen examination, number of slides investigated and criteria when a marker was called positive or negative should be provided.

6. Describe the number of independent (blinded) scorers and how they scored

Visual assessment of a biomarker is an important source of variance.[5] Interpretation varies between pathologists and biomarker data will be more robust if expression of a biomarker is scored by multiple independent observers unaware of (blinded to) the clinical parameter of interest (such as outcome). Justification of the chosen method of and criteria for (semi-) quantitative assessment should be provided in detail.

7. State the method of case selection, study design, origin of the cases and time-frame.

Important determinants of the reliability of study results are study design and method of patient selection. Selection of cases according to clinical or pathological parameters (for example patients selected according to age, only T4 or N0 tumors) may introduce bias; therefore details of case selection should be reported. Stating where the patients came from might provide relevant information regarding the patient population (for example a patient population from a tertiary referral hospital might differ significantly from that of a primary care centre). The time-frame (when cases were recruited or diagnosis was made) should also be mentioned, because therapies change over time which might affect outcome.

8. State the end of follow-up period and median follow up time.

In many studies outcome is the time to an event (e.g. recurrence, death), and follow-up should be long enough to make sure that events can happen. If for example a

biomarker is associated with the risk of dissemination, follow-up should be long enough to allow this effect to be observed. Follow-up usually ends at a specific point in time (notably this date and the median follow-up time should be stated).

9. Define all clinical endpoints examined.

In histopathology studies common endpoints include death and discovery of recurrence. Endpoints used in survival analysis are not always clearly defined. Analysis of time to death might include deaths from any cause or cancer specific deaths. A clear distinction should be made between overall survival, disease specific survival and recurrence free survival. Definition of parameters defining recurrence of disease should be clear. Recurrence might include local recurrence or distant metastasis or both. Local recurrence and distant metastases are two biologically different events and the effect of a biomarker on each of these might be different. Lack of clearly defined endpoints may lead to misinterpretation of its association with a biomarker and preclude inclusion of a publication in a meta-analysis.

10. Specify all applied statistical methods.

If the statistical methods used in a biomarker study are not clearly specified, it will be difficult or impossible for the reader to interpret the results or reproduce and validate the findings. Rather often the amount of detail provided in publications is marginal. Mathoulin-Pelissier et al. concluded that 68% of the articles published in major journals reported insufficient information regarding the survival analysis.[7]

11. Describe how interactions with other clinical/pathological variables were analyzed.

Any seemingly interesting biomarker might interact with established clinical or pathological factors. Methods used to assess potential interactions with other variables should be described. The interactions are essential to evaluate whether or not found associations have independent value. All included variables should be clearly defined and the choice of variables included in the study has to be justified (why variables included in the study were retained while others were left out).

12. Describe the number of patients included in the analysis and reasons for drop-out.

In retrospective biomarker studies the number of cases included in analysis is often lower than the initial number of cases included in the study. This is mainly due to missing values, such as impossibility to (re-) evaluate staining results or missing outcome data. A solution often chosen is to restrict the analyses to samples with complete data. However, this may introduce selection bias when samples with

missing data are not typical for the whole study population. It is therefore necessary to state the number of patients and events included in each analysis. Only with this information it is possible to assess the reliability of reported findings.

13. Report patient/disease characteristics (including the biomarker of interest) with number of missing values.

A detailed description of patient characteristics and relevant histopathological parameters is needed to assess whether or not the patient cohort included in the study is representative for the condition under scrutiny. Obvious patient characteristics are age and gender but parameters such as ethnicity, performance status or medical history might be relevant. In case of cancer, characteristics of the lesion should include parameters defining TNM stage.

14. Describe the interaction of the factor of interest with established prognostic variables

As stated in point 11, a new biomarker is only useful if its effect is maintained when interaction with other prognostic factors is ruled out, or if its assessment is (quantitatively or qualitatively) superior in comparison with established prognostic variables. For evaluation of clinical value, the potential interactions between a new biomarker and established prognostic variables should therefore be reported.

15. Included at least 90% of initial cases in univariate and multivariate analysis

As mentioned above, due to missing values the number of cases included in statistical analysis is often lower than the initial number of cases included in the study. The risk of attrition bias will increase along with the proportion of cases not included in statistical analysis[6] To minimize attrition bias, Smith et al. proposed that at least 90% of the selected cohort should be included in statistical analysis.[8] Sub-analyses should be avoided because of the high risk of false positive findings due to increasingly small patient numbers.

16. Report the estimated effect (relative risk/odds ratio, confidence interval and p-value provided) of the biomarker in univariate analysis

Establishing a biomarker's potential association with clinical outcome is the key subject in biomarker research. In univariate analysis the relationship between the biomarker and outcome can be assessed without adjustment for additional variables. Relative risks or odds ratios with their associated confidence intervals and p-values should be given, regardless of statistical significance. Kaplan-Meier curves should be included when illustrative, but p-values from log rank tests should be given regardless

of statistical significance. Univariate analysis should also be performed for all other variables and presented in a summarizing table.

17. Report the estimated effect (hazard ratio, confidence interval and p-value provided) of the biomarker in multivariate analysis.

In multivariate analysis the association between a biomarker and clinical outcome can be established, correcting for established prognostic variables. Authors should report which prognostic variables were included in multivariate analysis. As a rule, significant factors identified in univariate analysis should all be included. Hazard ratios with associated confidence intervals and p-values should be given, regardless of statistical significance.

18. Report estimated effects (hazard ratio, confidence interval and p-values provided) of other prognostic factors included in multivariate analysis.

Within a study significant findings are more likely to be reported than non-significant findings. In order to prevent selective reporting bias, authors should report the effects of all prognostic factors included in the multivariable analysis; not only the marker of interest or the significant findings.

19. Interpret the results in the context of the working hypothesis elaborated in the introduction and other relevant studies; include a discussion of limitations of the study.

Authors should critically evaluate their findings, mentioning limitations of the study and possible biases. A good discussion will allow the reader to retain a balanced perception of the importance of the results of the study.

20. Discuss potential clinical applications and implications for future research.

The intention of biomarker studies is to develop new disease associated parameters of which the contribution to clinical decision making reaches beyond that of existing parameters included in standard of care. A statistically significant association between a marker and disease outcome might seem promising, but authors should mention in the discussion which steps will be taken in order to eventually reach implementation of the marker in patient care.

Conclusion

Adherence to guidelines on reporting, whenever possible, should facilitate a clear perception by the reader of the inherent qualities of the reported study and we presume that it might also have a positive effect on study quality, for as much as the checkpoints we propose are already used when the study is planned. The 20 checkpoints we propose speak for themselves. We paid no attention to sample size calculations, because most histopathological studies are retrospective and based upon convenience case collections that were not set up to answer specific questions well defined before the collection was started. Checking model assumptions, standardized model making and model validation is unusual in histopathology research but might become more main-stream when this is more often performed in the context of clinical trials. For a biomarker identified in a retrospective study we consider external validation by independent groups on separate patient cohorts of much greater value than internal validation. Our checkpoints might be of help for investigators who study tissue-based biomarkers, reviewers of manuscripts and researchers performing meta-analyses. They should ultimately support quality improvement of histopathological studies and implementation of new findings into daily practice. We welcome feedback from the scientific community to improve on and facilitate implementation of our list of checkpoints.

References

1. Deutsch EW, Ball CA, Berman JJ, Bova GS, Brazma A, Bumgarner RE, Campbell D, Causton HC, Christiansen JH, Daian F, Dauga D, Davidson DR, Gimenez G, Goo YA, Grimmond S, Henrich T, Herrmann BG, Johnson MH, Korb M, Mills JC, Oudes AJ, Parkinson HE, Pascal LE, Pollet N, Quackenbush J, Ramialison M, Ringwald M, Salgado D, Sansone SA, Sherlock G, Stoeckert CJ, Jr., Swedlow J, Taylor RC, Walashek L, Warford A, Wilkinson DG, Zhou Y, Zon LI, Liu AY, True LD (2008) Minimum information specification for in situ hybridization and immunohistochemistry experiments (MISFISHIE). Nature biotechnology 26 (3):305-312. doi:10.1038/nbt1391
2. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, Statistics Subcommittee of the NCIEWGoCD (2005) Reporting recommendations for tumor marker prognostic studies. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 23 (36):9067-9072. doi:10.1200/JCO.2004.01.0454
3. Knijn N, Nagtegaal ID (2014) Guidelines for reporting histopathology studies. Journal of clinical pathology 67 (12):1111-1112. doi:10.1136/jclinpath-2014-202647
4. Khleif SN, Doroshow JH, Hait WN, Collaborative A-F-NCB (2010) AACR-FDA-NCI Cancer Biomarkers Collaborative consensus report: advancing the use of biomarkers in cancer drug development. Clinical cancer research : an official journal of the American Association for Cancer Research 16 (13):3299-3318. doi:10.1158/1078-0432.CCR-10-0880
5. True LD (2014) Methodological requirements for valid tissue-based biomarker studies that can be used in clinical practice. Virchows Archiv : an international journal of pathology 464 (3):257-263. doi:10.1007/s00428-013-1531-0
6. Higgins JPT, Green S (2011) Cochrane Handbook for Systematic Reviews of Interventions. Version 5.1.0 [updated March 2011]. The Cochrane Collaboration. www.cochrane-handbook.org.
7. Mathoulin-Pelissier S, Gourgou-Bourgade S, Bonnetain F, Kramar A (2008) Survival end point reporting in randomized cancer clinical trials: a review of major journals. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 26 (22):3721-3726. doi:10.1200/JCO.2007.14.1192
8. Smith RA, Tang J, Tudur-Smith C, Neoptolemos JP, Ghaneh P (2011) Meta-analysis of immunohistochemical prognostic markers in resected pancreatic cancer. British journal of cancer 104 (9):1440-1451. doi:10.1038/bjc.2011.110

Discussion

Adapted from: **Pathways of Spread in Rectal Cancer: A reappraisal of the True Routes to Distant Metastatic Disease.**
Lord AC, Knijn N, Brown G, Nagtegaal ID.

In preparation

Introduction

There have been major efforts to apply our ever expanding knowledge of molecular and cellular processes in colorectal cancer to gain insights into the metastatic process, however predicting which patient will develop metastases remains difficult. Thus, treatment decisions in colorectal cancer are generally based on TNM staging. With the increased resolution of imaging techniques in combination with high quality pathology, we can try to take the next step in colorectal cancer staging and appreciate the anatomical "highways" resulting in more accurate prediction of patient outcome, and critically re-appraise the traditional "lymph node–oriented" way of treatment decision making.

Although the TNM system[1] is currently the best tool for predicting prognosis, some issues need to be addressed.[2, 3] The main driver for prognosis is the presence/absence of regional lymph node metastases (LNM), but overlap between stage II and stage III patients (i.e. stage IIb patients perform worse than stage IIIa patients)[4] suggests that the importance of LNM is limited. Moreover, the presence of venous and lymphatic invasion (LI), perineural invasion (PNI) and extranodal tumor deposits (TD) are not separately classified in the TNM system, which inevitably results in a loss of the prognostic power. Growing evidence now indicates that for an adequate staging system these morphologic features should be appropriately weighed to improve prognostic accuracy.

This review aims to critically synthesise the evidence for each mode of tumor spread in order to improve the prognostic value of staging and resulting treatment decisions.

Routes of spread

Direct invasion

Depth of spread into and beyond the bowel wall is directly linked to prognosis and forms an important feature in staging systems.[5-7] I believe that depth of invasion is important in the pathogenesis of metastatic disease for two reasons: firstly, with increasing depth of invasion, the likelihood of the tumor encountering and invading an anatomical highway also increases, leading to systemic spread. Secondly, locally advanced tumors can threaten the circumferential resection margin (CRM) in rectal cancer, or can directly invade adjacent organs or can spread within the peritoneal cavity. The depth of invasion can be seen on pathology and – in case of rectal cancer - on staging MRI with a high level of prognostic accuracy.[8] This information is used to plan the sequence and contents of treatment and indicates whether neoadjuvant therapy is necessary for tumor downstaging in rectal cancer[8] or whether local excision[9] is sufficient in case of early rectal cancer.

Lymphatic invasion and lymph node metastases

Tumor infiltration into the local lymphatics (lymphatic invasion (LI)) may be associated with a poor prognosis (Figure 1) but its individual contribution is unclear because many studies have grouped lymphatic and venous invasion as a single entity "lymphovascular spread". However, we consider this incorrect since studies in which lymphatic spread is described separately its prognostic relevance is much less important than venous invasion.[10, 11] Lymphatic spread is closely linked to lymph node metastasis, as one would expect.[12-14] Lymphatic and venous channels are completely different pathways of spread and should therefore always be reported as distinct entities.

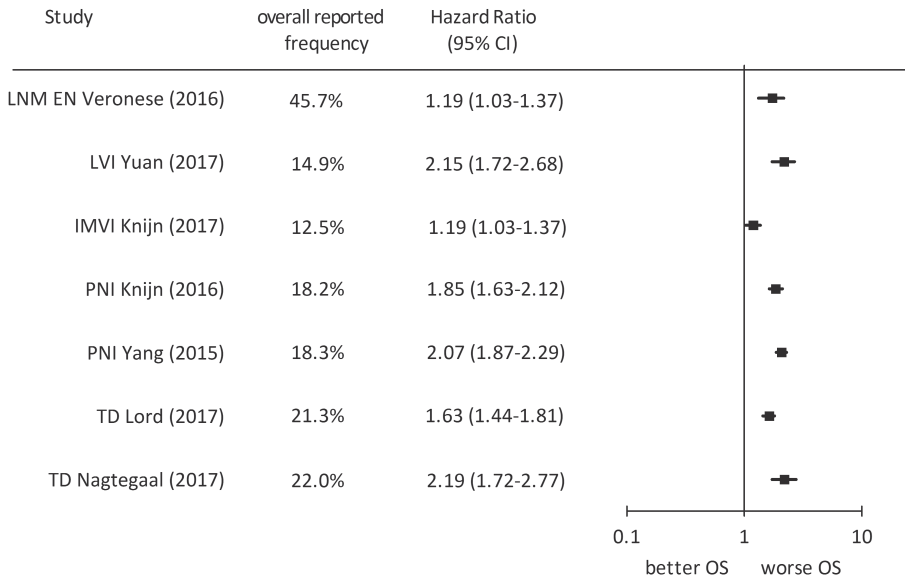


Figure 1. LNM : lymph node metastases; EN: extra nodal growth; LVI: lymphovascular invasion; IMVI: intramural vascular invasion; PNI: perineural invasion; TD: tumor deposits; CI: confidence interval; OS: overall survival.

Lymph node involvement is traditionally considered as the most important feature of a high-risk tumor and is one of the most common indications for neo-adjuvant and adjuvant therapy. Lymph node metastases can be detected on MRI (indication for neo-adjuvant therapy) or, most reliably, during pathology investigation (indication for adjuvant chemotherapy) (Figure 2).

Two histological features have additional value at the histological evaluation of lymph node metastases: micrometastases and extranodal growth. Small clusters of tumor cells, detected within a lymph node, using molecular detection techniques or

immunohistochemistry are – depending on their size – classified as micrometastases (between 0.02 and 0.2 cm in diameter) or isolated tumor cells (single or small nest of distinct tumor cells smaller than 0.02 cm, only detectable with IHC or molecular biology).[1, 15] Their presence is associated with worse outcome in meta-analyses compared to negative lymph nodes (Figure 1).[16, 17] If the metastatic tumor cells extend through the nodal capsule into the perinodal fat (extranodal growth), this is associated with a worse prognosis compared to positive lymph nodes without extranodal growth.[18]

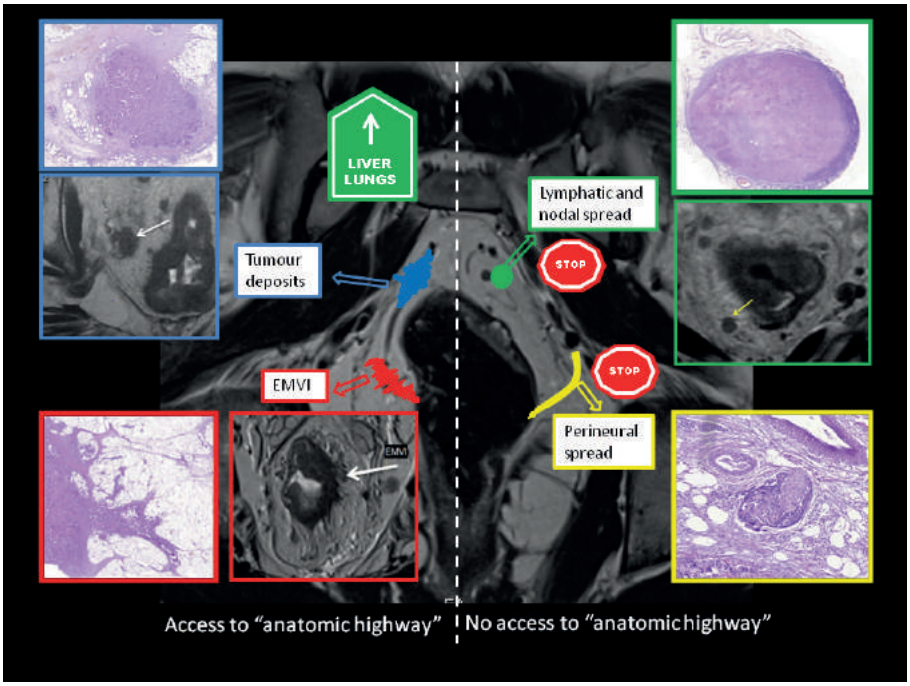


Figure 2. EMVI: Extramural vascular invasion

Much attention is focused on lymph node yield, since increasing numbers of examined lymph nodes are associated with improved outcome.[19, 20] This could be due to a more radical surgical technique with removal of sufficient mesorectal and mesocolic fat associated with subsequent improved oncological outcome and/or to the effect of upstaging patients who subsequently benefit from additional adjuvant treatment.[21, 22] However a high number of lymph nodes could also be the result of an ongoing immune response, which in itself confers a survival advantage.[22-24] Some studies have shown that the use of positive: negative lymph node ratio rather than total number of involved lymph nodes is a better predictor of prognosis than traditional N stage,[25-27] since this might indeed reflect the power of the immune response.

A mechanistic view of the TNM system has often been taken in that the tumor first seeds to lymph nodes, which in turn cause distant metastases. This does not make anatomical sense, as there is no direct access from the lymphatic system to the portal circulation, as one would expect given that the liver is the most common site of metastases. Furthermore we found no association with lymph node status in patients with liver and lung metastases, providing indirect evidence for an alternative metastatic pathway, most likely vascular.[28] A recent study by Naxerova et al supports this hypothesis, by showing 65% discordance in the subclonal origin of lymph node and distant metastases.[29]

With the widespread adoption of high quality TME surgery, it has been suggested that lymph nodes are no longer an important cause of local recurrence unless they are outside the TME plane.[30] Some studies have gone further and shown that there is no strong link between lymph node involvement and distant metastases.[28, 31] This may account for the lack of significant improvements in survival as observed with the use of adjuvant chemotherapy in lymph node positive patients.[32]

Venous invasion, both intramural and extramural

Venous invasion has consistently been shown to be associated with poor prognosis, both when detected by pathology[33-35] and MRI.[35-39] It has a particularly strong association with synchronous metastases, which is stronger than that of nodal disease.[38] The direct access to the portal circulation in tumors drained by the inferior mesenteric vein supports this association since direct venous dissemination to the liver may be more successful than indirect dissemination via the lymphatic system. One could hypothesise that low rectal tumors drained by the inferior rectal veins, and therefore the systemic rather than portal circulation, may be more closely associated with lung rather than liver metastases, but conclusive evidence to support this hypothesis is lacking. Distinction has been made between large vein and small vein EMVI and also between intramural and extramural venous spread. Extramural spread appears to be more strongly associated with a poor prognosis, as is the invasion of larger veins.[10, 11] Nevertheless, intramural invasion is a poor prognostic marker (Figure 1).[40]

Several authors have highlighted the problems with underreporting of EMVI on pathology, which makes it more difficult to assess its prognostic effect.[10, 11, 41, 42] The use of elastin staining greatly improves EMVI detection but this is not commonly used in practice.[42-45] The Royal College of Pathologists states that EMVI detection rate should be greater than 30% as this is a useful quality marker. In our experience, the reported prevalence is often lower and this might be improved by using MRI scans to alert the pathologist to the presence of EMVI. EMVI is seen more readily on MRI than on pathology (Figure 2) and the use of MRI detected EMVI as a prognostic marker has previously been validated.[37, 39]

Tumor Deposits; Origin and Impact

Tumor deposits (TD) are defined as "separate nodules or deposits of malignant cells in the perirectal or pericolic fat without evidence of residual lymph node tissue" (Figure 2).[1] They have only recently been recognised as a prognostically distinct entity in TNM 7 and 8. In previous versions these were classified as either lymph nodes or as part of the T stage according to inconsistent criteria which has resulted in controversy within the pathology community. In the current TNM edition these are classified as "N1c", the implication being that their prognostic effect is somewhere between having involvement of 3 and 4 lymph nodes. This is, however, not evidence based as two recent meta-analyses[46, 47] have shown that the presence of TD is actually worse (i.e. more in line with N2 or even M1 disease).

There are problems with the consistent pathological reporting of TD, partly due to lack of clarity in diagnostic criteria and a great degree of inter-observer variability, and partly due to differences in TNM staging systems. This is shown by the large variation in prevalence (10%-40%) reported in meta-analyses (Figure 1).[46, 47] In TNM 5 TD should be reported as lymph node metastases or as part of the T stage depending on their size. Even in TNM 7 and 8 there is no requirement to report TD in patients who also have lymph node metastases as this latter is considered to be of more importance (an assumption we strongly question given recent data).[47] We conclude that it is impossible to individually evaluate the prognostic effect of lymph node metastases versus TD, as long as TD are not separately classified.

Perineural invasion

PNI is defined as neoplastic invasion of nervous structures with spread along nerve sheaths (Figure 2). The presence of PNI has been consistently shown to be a poor prognostic marker (Figure 1).[48, 49] There is a great variation in reported prevalence, ranging from 2% to over 50%.[48, 49] S100 staining has been shown to significantly increase detection but is not used as standard. Some studies have shown that the severity of PNI gives further prognostic information[50] although this is not commonly reported. Although tumor growing along nerves would logically provide a route to local spread, threatening the CRM and contributing to the risk of local recurrence, the route to causing distant metastatic disease is unclear. We would hypothesise that PNI is more likely an indicator of an aggressive tumor with metastatic potential and a possible cause of local recurrence, rather than a direct cause of distant metastases. There is evidence to support this hypothesis.[51]

Combination of spread: limited evidence

Given that many of these pathways of spread are present simultaneously, it is impossible on the basis of current evidence to separate out the prognostic effect of

each parameter individually. Meta-analyses have shown that each feature confers a survival disadvantage but this is generally on univariate analysis and does not tell us whether a radiological or pathological feature is a bystander or the true cause of spread. Patients with lymph node metastases have a worse prognosis than those without, but most patients with lymph node metastases also have EMVI, so was EMVI or lymph node metastases the cause of distant metastases?

Conclusions

Assessment of individual prognostic features is currently made difficult by variations in the quality of pathological assessment and interobserver variation, as demonstrated by the significant differences in the reported prevalence of these features between studies.[10, 41, 46, 47] Multiple authors have shown that additional techniques such as elastin staining and immunohistochemistry can greatly increase the detection rate of features such as EMVI and PNI.[2, 10, 41] Further prospective work is needed with meticulous pathological assessment and reporting to ensure adequate detection rates of features such as EMVI and PNI, separate out venous from lymphatic invasion and TD from lymph node metastases. Problems such as under-detection of EMVI and misclassification of TD implies that retrospective data may be flawed, and that any conclusions need to be reconsidered.

It is of paramount importance that a distinction is made between two categories of radiological and pathological tumor spread: tumor that has spread locally but does not have the ability to cause distant metastases versus tumor that has the ability of dissemination irrespectively of local extent. The first category includes lymph node metastases, LI and PNI which are biomarkers of an aggressive tumor with the ability to spread but, as long as they can be removed with adequate surgery, do not have access to the anatomical "highways" which lead to metastases. The second category predominantly centres on venous invasion, as this is how tumor accesses the circulation. Probably TD should be included in this category as well, since MRI imaging suggests they are closely related to veins and are, in many cases, likely to be a result of discontinuous EMVI with formation of nodules along the course of a vein. This hypothesis requires validation which is planned in a prospective trial.[52]

The widespread availability of technology today means that it would be incredibly easy for clinicians to access a multivariable algorithm via PCs, tablets or smartphones. This is quite common in other situations in medicine such as calculating POSSUM scores to assess pre-operative risk. However, we have still not seized the opportunity that technology gives us to develop more prognostically accurate staging algorithms. In our opinion this is a missed opportunity to deliver personalised cancer care. It

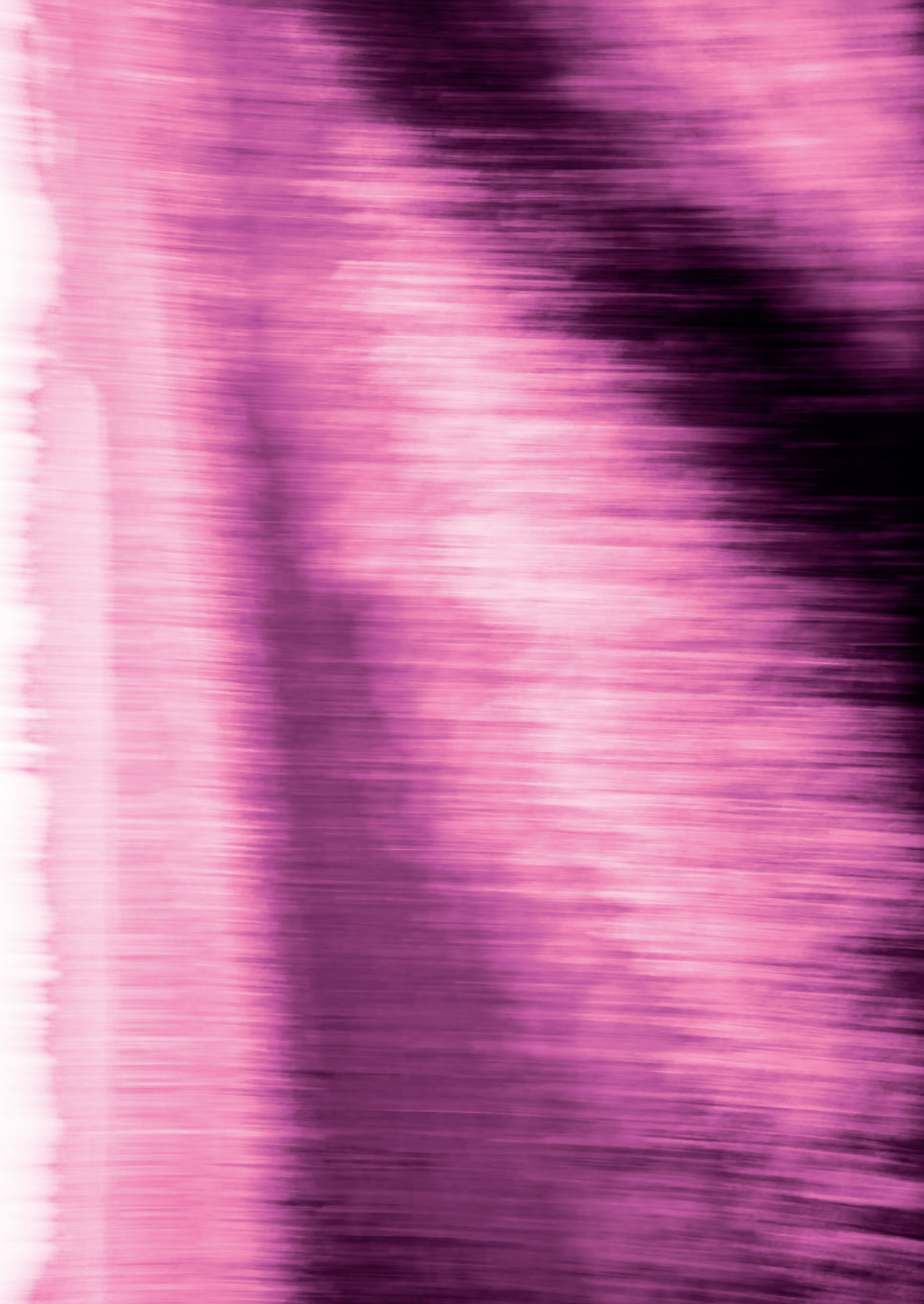
is possible that molecular and genetic factors could also be taken into account and may add additional prognostic information.[53-55] Novel techniques such as the measurement of circulating tumor DNA or other biomarkers may also be useful in the future but are still at an early stage of investigation.[56] We suggest that the TNM system needs further improvement, as it does not accurately predict prognosis. TNM 7 and 8 have increased in complexity with multiple sub-classifications, but remain over-simplistic in ignoring important routes of local spread other than crudely assessed tumor depth and LN metastases. We recommend that the following factors are taken into account in any future staging system: depth of invasion, LN status, EMVI, TD, lymphatic invasion, perineural invasion, CRM involvement and tumor height. There is an urgent need to improve the pathological and radiological documentation of these features in routine practice, given their potential prognostic effect and the ability to personalise outcome. Greater precision in documentation of these features will, in the future, yield a better understanding of the real drivers of metastatic spread.

References

1. Sobin, L.H., M.K. Gospodarowicz, and C. Wittekind, *TNM classification of malignant tumours*. 7th ed. ed. 2010, Oxford: Wiley-Blackwell.
2. Maguire, A. and K. Sheahan, *Controversies in the pathological assessment of colorectal cancer.* World J Gastroenterol, 2014. **20**(29): p. 9850-61.
3. Nagtegaal, I.D., P. Quirke, and H.J. Schmoll, *Has the new TNM classification for colorectal cancer improved care?* Nat Rev Clin Oncol, 2011. **9**(2): p. 119-23.
4. Nitsche, U., et al., *Prediction of prognosis is not improved by the seventh and latest edition of the TNM classification for colorectal cancer in a single-center collective.* Ann Surg, 2011. **254**(5): p. 793-800; discussion 800-1.
5. Astler, V.B. and F.A. Coller, *The prognostic significance of direct extension of carcinoma of the colon and rectum.* Ann Surg, 1954. **139**(6): p. 846-52.
6. Kirklin, J.W., M.B. Dockerty, and J.M. Waugh, *The role of the peritoneal reflection in the prognosis of carcinoma of the rectum and sigmoid colon.* Surg Gynecol Obstet, 1949. **88**(3): p. 326-31.
7. Sobin, L.H., P. Hermanek, and R.V. Hutter, *TNM classification of malignant tumors. A comparison between the new (1987) and the old editions.* Cancer, 1988. **61**(11): p. 2310-4.
8. Taylor, F.G., et al., *Preoperative magnetic resonance imaging assessment of circumferential resection margin predicts disease-free survival and local recurrence: 5-year follow-up results of the MERCURY study.* J Clin Oncol, 2014. **32**(1): p. 34-43.
9. Balyasnikova, S. and G. Brown, *Optimal Imaging Strategies for Rectal Cancer Staging and Ongoing Management.* Curr Treat Options Oncol, 2016. **17**(6): p. 32.
10. Betge, J. and C. Langner, *Vascular invasion, perineural invasion, and tumour budding: predictors of outcome in colorectal cancer.* Acta Gastroenterol Belg, 2011. **74**(4): p. 516-29.
11. Betge, J., et al., *Intramural and extramural vascular invasion in colorectal cancer: prognostic significance and quality of pathology reporting.* Cancer, 2012. **118**(3): p. 628-38.
12. Glasgow, S.C., et al., *Meta-analysis of histopathological features of primary colorectal cancers that predict lymph node metastases.* J Gastrointest Surg, 2012. **16**(5): p. 1019-28.
13. Akagi, Y., et al., *Prognostic impact of lymphatic invasion of colorectal cancer: a single-center analysis of 1,616 patients over 24 years.* Anticancer Res, 2013. **33**(7): p. 2965-70.
14. Bosch, S.L., et al., *Predicting lymph node metastasis in pT1 colorectal cancer: a systematic review of risk factors providing rationale for therapy decisions.* Endoscopy, 2013. **45**(10): p. 827-34.
15. Hermanek, P., et al., *International Union Against Cancer. Classification of isolated tumor cells and micrometastasis.* Cancer, 1999. **86**(12): p. 2668-73.
16. Iddings, D., et al., *The prognostic effect of micrometastases in previously staged lymph node negative (N0) colorectal carcinoma: a meta-analysis.* Ann Surg Oncol, 2006. **13**(11): p. 1386-92.
17. Sloothaak, D.A., et al., *The prognostic value of micrometastases and isolated tumour cells in histologically negative lymph nodes of patients with colorectal cancer: a systematic review and meta-analysis.* Eur J Surg Oncol, 2014. **40**(3): p. 263-9.
18. Veronese, N., et al., *Prognostic impact and implications of extracapsular lymph node involvement in colorectal cancer: a systematic review with meta-analysis.* Ann Oncol, 2016. **27**(1): p. 42-8.
19. Chang, G.J., et al., *Lymph node evaluation and survival after curative resection of colon cancer: systematic review.* J Natl Cancer Inst, 2007. **99**(6): p. 433-41.
20. Ong, M.L. and J.B. Schofield, *Assessment of lymph node involvement in colorectal cancer.* World J Gastrointest Surg, 2016. **8**(3): p. 179-92.
21. Feinstein, A.R., D.M. Sosin, and C.K. Wells, *The Will Rogers phenomenon. Stage migration and new diagnostic techniques as a source of misleading statistics for survival in cancer.* N Engl J Med, 1985. **312**(25): p. 1604-8.

22. George, S., et al., *Will Rogers revisited: prospective observational study of survival of 3592 patients with colorectal cancer according to number of nodes examined by pathologists.* Br J Cancer, 2006. **95**(7): p. 841-7.
23. Pihl, E., et al., *Lymphoid hyperplasia: a major prognostic feature in 519 cases of colorectal carcinoma.* Am J Pathol, 1980. **100**(2): p. 469-80.
24. Markl, B., *Stage migration vs immunology: The lymph node count story in colon cancer.* World J Gastroenterol, 2015. **21**(43): p. 12218-33.
25. Berger, A.C., et al., *Colon cancer survival is associated with decreasing ratio of metastatic to examined lymph nodes.* J Clin Oncol, 2005. **23**(34): p. 8706-12.
26. Zhang, J., et al., *Comparison of metastatic lymph node ratio staging system with the 7th AJCC system for colorectal cancer.* J Cancer Res Clin Oncol, 2013. **139**(11): p. 1947-53.
27. Parnaby, C.N., et al., *Prognostic value of lymph node ratio and extramural vascular invasion on survival for patients undergoing curative colon cancer resection.* Br J Cancer, 2015. **113**(2): p. 212-9.
28. Knijn, N., et al., *Limited effect of lymph node status on the metastatic pattern in colorectal cancer.* Oncotarget, 2016. **7**(22): p. 31699-707.
29. Naxerova, K., et al., *Origins of lymphatic and distant metastases in human colorectal cancer.* Science, 2017. **357**(6346): p. 55-60.
30. Chand, M., et al., *Lymph node status does not predict local recurrence in the total mesorectal excision era.* Dis Colon Rectum, 2014. **57**(1): p. 127-9.
31. Enquist, I.B., et al., *Lymph node-independent liver metastasis in a model of metastatic colorectal cancer.* Nat Commun, 2014. **5**: p. 3530.
32. Petersen, S.H., et al., *Postoperative adjuvant chemotherapy in rectal cancer operated for cure.* Cochrane Database Syst Rev, 2012(3): p. CD004078.
33. Courtney, E.D., et al., *Extramural vascular invasion is an adverse prognostic indicator of survival in patients with colorectal cancer.* Colorectal Dis, 2009. **11**(2): p. 150-6.
34. McClelland, D. and G.I. Murray, *A Comprehensive Study of Extramural Venous Invasion in Colorectal Cancer.* PLoS One, 2015. **10**(12): p. e0144987.
35. Chand, M., et al., *Systematic review of prognostic importance of extramural venous invasion in rectal cancer.* World J Gastroenterol, 2016. **22**(4): p. 1721-6.
36. Chand, M., et al., *The prognostic significance of postchemoradiotherapy high-resolution MRI and histopathology detected extramural venous invasion in rectal cancer.* Ann Surg, 2015. **261**(3): p. 473-9.
37. Chand, M., et al., *Extramural venous invasion is a potential imaging predictive biomarker of neoadjuvant treatment in rectal cancer.* Br J Cancer, 2014. **110**(1): p. 19-25.
38. Siddiqui, M.R.S., et al., *A meta-analysis comparing the risk of metastases in patients with rectal cancer and MRI-detected extramural vascular invasion (mrEMVI) vs mrEMVI-negative cases.* Br J Cancer, 2017. **116**(12): p. 1513-1519.
39. Smith, N.J., et al., *Prognostic significance of magnetic resonance imaging-detected extramural vascular invasion in rectal cancer.* Br J Surg, 2008. **95**(2): p. 229-36.
40. Knijn, N., et al., *The value of intramural vascular invasion in colorectal cancer - a systematic review and meta-analysis.* Histopathology, 2017.
41. Messenger, D.E., D.K. Driman, and R. Kirsch, *Developments in the assessment of venous invasion in colorectal cancer: implications for future practice and patient outcome.* Hum Pathol, 2012. **43**(7): p. 965-73.
42. Kingston, E.F., H. Goulding, and A.C. Bateman, *Vascular invasion is underrecognized in colorectal cancer using conventional hematoxylin and eosin staining.* Dis Colon Rectum, 2007. **50**(11): p. 1867-72.
43. Howlett, C.J., E.J. Tweedie, and D.K. Driman, *Use of an elastic stain to show venous invasion in colorectal carcinoma: a simple technique for detection of an important prognostic factor.* J Clin Pathol, 2009. **62**(11): p. 1021-5.

44. Baumhoer, D., et al., *Impact of using elastic stains for detection of venous invasion in the prognosis of patients with lymph node negative colorectal cancer.* Int J Colorectal Dis, 2010. **25**(6): p. 741-6.
45. Inoue, T., et al., *Vascular invasion of colorectal carcinoma readily visible with certain stains.* Dis Colon Rectum, 1992. **35**(1): p. 34-9.
46. Lord, A.C., et al., *Significance of extranodal tumour deposits in colorectal cancer: A systematic review and meta-analysis.* Eur J Cancer, 2017. **82**: p. 92-102.
47. Nagtegaal, I.D., et al., *Tumor Deposits in Colorectal Cancer: Improving the Value of Modern Staging-A Systematic Review and Meta-Analysis.* J Clin Oncol, 2017. **35**(10): p. 1119-1127.
48. Knijn, N., et al., *Perineural Invasion is a Strong Prognostic Factor in Colorectal Cancer: A Systematic Review.* Am J Surg Pathol, 2016. **40**(1): p. 103-12.
49. Yang, Y., et al., *Prognostic value of perineural invasion in colorectal cancer: a meta-analysis.* J Gastrointest Surg, 2015. **19**(6): p. 1113-22.
50. Ceyhan, G.O., et al., *The severity of neural invasion is a crucial prognostic factor in rectal cancer independent of neoadjuvant radiochemotherapy.* Ann Surg, 2010. **252**(5): p. 797-804.
51. Horn, A., O. Dahl, and I. Morild, *Venous and neural invasion as predictors of recurrence in rectal adenocarcinoma.* Dis Colon Rectum, 1991. **34**(9): p. 798-804.
52. Clinicaltrials.gov, *Concordance in MRI and Pathology Diagnosis of Extranodal Tumour Deposits.* Available from: https://clinicaltrials.gov/ct2/show/NCT03303547.
53. Xu, F., et al., *Classification based on the combination of molecular and pathologic predictors is superior to molecular classification on prognosis in colorectal carcinoma.* Clin Cancer Res, 2007. **13**(17): p. 5082-8.
54. Lea, D., et al., *Accuracy of TNM staging in colorectal cancer: a review of current culprits, the modern role of morphology and stepping-stones for improvements in the molecular era.* Scand J Gastroenterol, 2014. **49**(10): p. 1153-63.
55. Cremolini, C., et al., *FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab as first-line treatment of patients with metastatic colorectal cancer: updated overall survival and molecular subgroup analyses of the open-label, phase 3 TRIBE study.* Lancet Oncol, 2015. **16**(13): p. 1306-15.
56. Phallen, J., et al., *Direct detection of early-stage cancers using circulating tumor DNA.* Sci Transl Med, 2017. **9**(403).



Summary

Dissemination of colorectal cancer

Despite intensive follow-up and increasing therapeutic options for colorectal cancer (CRC), metastatic disease is the leading factor in CRC mortality. Tumor cells from CRC can disseminate to other parts of the body through different pathways. They can invade blood vessels, lymphatic vessels and lymph nodes, grow along nerves (perineural invasion) or directly grow through the bowel wall and reach the peritoneal cavity. Perineural invasion might be important in local spread, vascular and lymphatic invasion might be more important in dissemination towards distant organs. Direct growth of the tumor through the bowel wall is considered the key element for dissemination within the peritoneal cavity. Insight in the role of histological and molecular factors in the dissemination of CRC is lacking.

In **chapter 1** we reviewed the literature regarding prognostic histological features in colorectal liver metastases. As increasing numbers of patients are selected for resection of colorectal liver metastases, it is crucial to identify factors that may predict outcome after resection. This review shows that invasion of tumor cells in the portal vein and in lymphatic vessels are related to prognosis. Moreover a fibrous capsule around the metastases appears to be correlated with prognosis. The involvement of resection margins is a strong prognostic factor, although the significance of the width of negative margins remains controversial. Various studies have evaluated tumor response to neo-adjuvant chemotherapy, but different grading systems were used, and definite recommendations cannot be made. Research of colorectal liver metastases is still in its infancy, and well-defined factors, studied in homogenous patient populations are needed to improve patient care. That is the reason why we addressed some of the suggested prognostic factors from literature in a homogenous patient population in **chapter 2.** We investigated possible prognostic histologic factors in 124 patients who underwent a complete resection of solitary colorectal liver metastases without neo-adjuvant treatment. We evaluated tumor thickness at the tumor-normal interface, the presence of a fibrotic capsule, intrahepatic vascular invasion, lymphatic invasion, or bile duct invasion and perineural invasion, using immunohistochemistry. All variables that were associated with survival in univariate analysis were included in multivariate analysis. There was no association between histologic factors and disease-free survival in multivariate analysis. Intrahepatic lymphatic invasion was associated with a decreased overall survival in multivariate analysis, especially in combination with vascular invasion. Size of the metastases over 50 mm and an interval of less than 12 months between resection of the primary tumor and diagnosis of liver metastasis were other independent adverse prognostic factors. We concluded that only intrahepatic lymphatic invasion, especially in combination with vascular invasion, is an important adverse prognostic factor for

overall survival in patients with resected colorectal liver metastases. Therefore, we recommend to evaluate the presence or absence of intrahepatic lymphatic and vascular invasion in the histological assessment of colorectal liver metastases. Future research is needed to determine whether adjuvant treatment strategies should be based on these adverse prognostic histologic factors.

Identification of mutations in the proto-oncogenes *KRAS* and *NRAS* as predictive markers for response to anti-EGFR therapy has improved patient selection, however even in *RAS* wild type patients response to anti-EGFR therapy is limited. *KRAS* mutation analysis is usually performed on primary tumor tissue, because metastatic tissue is not always available. A possible discordance of test results between primary tumors and metastases has been suggested as an explanation for the failure rate of anti-EGFR therapy in patients without mutations in *KRAS*. In **chapter 3** we evaluated the concordance of the *KRAS* mutation status in 305 primary colorectal tumors and their corresponding liver metastases. *KRAS* mutations were detected in 35.4% of primary tumors. In 11 cases (3.6%) we observed a discordance between primary tumors and metastases: 5 primary tumors had a *KRAS* mutation which was not found in the metastases, 1 primary tumor was wild type with a mutation in the metastases, and in 5 cases the primary tumor and metastases had a different type of *KRAS* mutation. In only 6 patients the discordance was clinically relevant (2.0%). In this large and homogeneous study, we observed a high concordance of *KRAS* mutation status of 96.4% (95%CI 93.6-98.2%) between primary colorectal tumors and corresponding liver metastases. Therefore, both primary tumors and liver metastases can be used for *KRAS* mutation analysis.

In **chapter 4** we have expanded our research on the concordance in mutational status between primary tumors and corresponding liver metastases to lung metastases. Next to *KRAS* mutation status, mutation status of the proto-oncogenes *BRAF, HRAS, NRAS* and *PIK3CA* were investigated using next-generation sequencing including single molecule tags. The obtained number of unique reads was not always sufficient to confidentially call the absence or presence of mutations for all regions of interest. Paired sequencing results on all five genes were reached in 249 of the 402 cases (62%). With this unique sequencing technique, we observed a high concordance in *RAS/RAF* mutation status between tumors and corresponding metastases, which implies that both primary tumors and their distant metastases can be used to determine the mutational status for targeted therapy. Lung metastases had a higher percentage of *RAS* mutations compared to liver metastases (71% vs. 48%). We showed that next-generation sequencing including a single molecule tags is feasible, however in combination with archival formalin-fixed paraffin embedded material is limited by coverage depth.

Nodules or foci of cancer cells can be found in the pericolic or perirectal fat, without evidence of residual lymph node tissue. Those nodules are called tumor deposits. The etiology of tumor deposits is unclear, and they are currently included in staging as a separate lymph node category (N1c). If tumor deposits are equal to lymph node metastases, both in prognostic and biological sense, this would simplify staging. However, if tumor deposits add information to staging, we should apply specific sub-staging. In **chapter 5** we investigated the prognostic value of tumor deposits in comparison to lymph node metastases. We assessed the prognostic value of tumor deposits in CRC by systematically reviewing the literature and performing a meta-analysis. Seventeen articles with a total of 10106 CRC patients were identified, and tumor deposits were observed in 22% of patients. Tumor deposits occurred more often in tumors with lymph node metastases and with extramural vascular invasion (EMVI). Tumor deposits showed a strong effect on disease-free, cancer specific and overall survival with a hazard ratio of 2. We used four large cohorts, comprising 4914 patients, to investigate the impact of tumor deposits on metastatic pattern and compared it with the impact of lymph node metastases and EMVI. Logistic regression revealed that both tumor deposits and lymph node metastases increases the risk on liver metastases, lung metastases and peritoneal metastases. However the risk on liver metastases is significantly higher in patients with both tumor deposits and lymph node metastases, compared to lymph node metastases alone. Moreover, EMVI is associated with an increased risk on liver and lung metastases, but seems not to be involved in peritoneal metastases. Our study shows that tumor deposits are not equal to lymph node metastases or EMVI with respect to biology and outcome. Separate scoring of tumor deposits provides additional information about prognosis and is important in metastatic patterns. We conclude that valuable prognostic information is lost when tumor deposits are allocated to nodal category .

In **chapter 6** we report on the effect of regional lymph node metastases on metastatic patterns in CRC. We compared lymph node negative CRC (N-) with lymph node positive CRC (N+) in a large autopsy study comprising 1393 patients with metastatic CRC. Incidences of peritoneal metastases and distant lymph node metastases were higher in the N+ group (28% vs. 21% and 25% vs. 15%). The incidence of liver and lung metastases was comparable in both groups. We validated our findings in a population-based study of 2382 patients of the Eindhoven Cancer Registry. Peritoneal metastases occurred in 22% of the N+ patients, compared to 17% of the N- patients. The incidence of distant lymph node metastases was 16% in the N+ group, compared to 10% in the N- group. The higher incidence of peritoneal and distant lymph node metastases, but comparable incidences of liver and lung metastases shows that lymph node metastases have a limited influence on metastatic patterns. We conclude that dissemination to distant organs, like the

liver and lung, occurs independently of lymphatic spread. Moreover, we assume that lymph node metastases function as a sign of advanced disease and are not involved in the metastatic process.

Tumor growth along nerves, perineural invasion (PNI), is described in many cancers. Its prognostic value in CRC is not established. In **chapter 7** we systematically reviewed the impact of PNI in CRC. We included 58 studies, comprising 22.900 patients, revealing an incidence of 18%. PNI is more often found in rectal tumors compared to colon tumors (22% vs. 16%) and is associated with tumor stage. Our meta-analysis confirms the strong impact of PNI on local recurrence, but also shows a strong prognostic impact on disease free survival, cancer specific and overall survival. The effect of PNI is comparable to that of established prognostic factors. Thus, PNI has a strong impact on local recurrence and survival, with a prognostic value similar to that of well-established prognostic factors. Therefore we recommend PNI to be included in the standardized reporting of CRC and might be considered a high-risk feature.

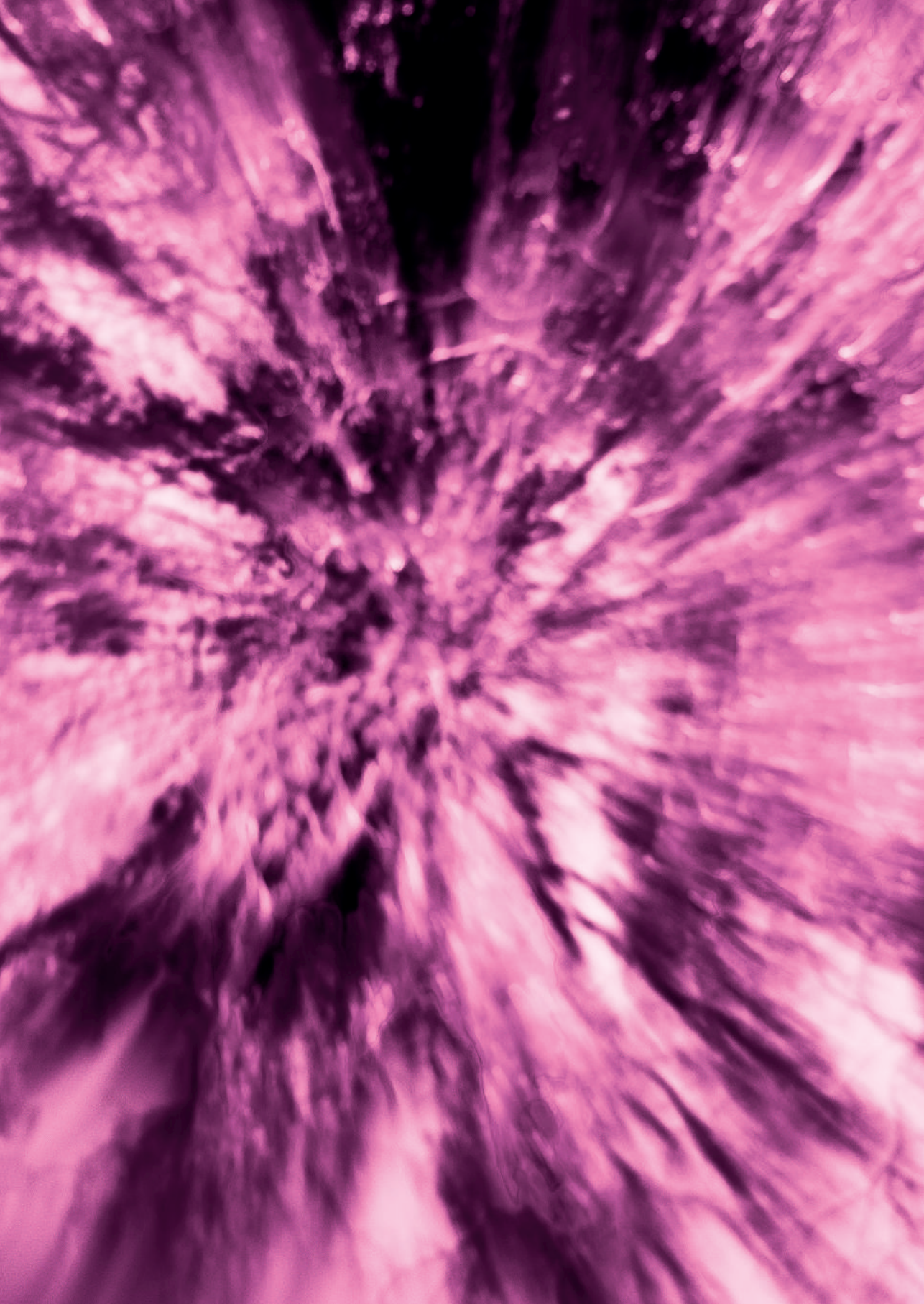
Vascular invasion is the presence of tumor cells within blood vessels. It can be subdivided into intramural vascular invasion (IMVI) and extramural vascular invasion (EMVI), according to the location of the vessels towards the muscle wall. EMVI is a well-established independent prognostic indicator, the prognostic importance of IMVI is less clear. In **chapter 8** we report the impact of IMVI in CRC. Our literature search revealed 20 articles, comprising 8078 patients. The overall reported incidence of IMVI is 12.5% and IMVI is associated with a decreased cancer specific survival. We conclude that, despite the limited number of studies, there is a clear association with outcome in the presence of IMVI. This warrants more attention to this underreported prognostic factor.

Most published histopathology studies are retrospective and this limits the quality of the evidence provided in those papers. A higher level of evidence, such as would be required to justify implementation in daily clinical practice, can be reached by systematic review of published studies and meta-analysis of the provided data. However, those meta-analyses are based on individual studies of which the quality of reporting varies. In **the appendix** we proposed a guideline to improve the reporting of retrospective histopathology studies. The intention of our proposal was to increase the awareness of the need for more standardization. Adherence to guidelines on reporting will support quality improvement of histopathological studies, facilitate comparison of results across different studies and ultimately help implementing new biomarkers into daily practice.

General conclusion

In this thesis we investigated the role of different histological and molecular features in the dissemination of colorectal cancer. We showed in colorectal liver metastases that invasion of tumor cells in lymphatic vessels within the liver is associated with a poorer survival. Reporting of this factor and other potentially prognostic factors in pathology reports of resected colorectal liver metastases may help to individualize patient care. We also analyzed factors in the primary tumor that might be associated with survival. Our meta-analyses provided evidence to incorporate perineural invasion and intramural vascular invasion as prognostic factors in the TNM staging system. Regional lymph node metastases play a crucial role in TNM staging and adjuvant treatment is based on the presence of lymph node metastases. However, we have challenged the role of lymph node metastases by demonstrating that other factors are more important in metastatic spread. We demonstrated the importance of tumor deposits and extramural vascular invasion in comparison to lymph node metastases. We advocate separate scoring of tumor deposits, as it provides additional information about prognosis and is important in metastatic patterns. Moreover, we showed that lymph node metastases have a limited influence on metastatic patterns. Lymph node metastases may herald more advanced disease, but are not involved in the metastatic process itself.

Next to prognostic factors, predictive factors are important to individualize patient care. In colorectal cancer the most important predictive marker is *RAS* mutation status for response to anti-EGFR therapy and therefore molecular testing is required to determine *RAS/RAF* mutation status prior to initiation of anti-EGFR therapy. The high concordance rate of *RAS* mutation status that we established between primary tumor and metastatic tissue justifies testing the primary tumor for anti-EGFR therapy. We excluded discordance in test results as explanation for the failure rate of anti-EGFR therapy in patients with tumors without mutations in *RAS*.



Nederlandse samenvatting

S

Metastasering van darmkanker

Ondanks vroege opsporing, verbeterde diagnostiek en toegenomen behandelopties voor darmkanker blijft gemetastaseerde ziekte een probleem. Er zijn verschillende routes van metastasering herkenbaar onder de microscoop: via bloedvaten, lymfevaten en lymfklieren, via zenuwen (perineurale invasie) of via directe groei door de darmwand. Het was onduidelijk welke rol de verschillende histologische en moleculaire factoren spelen bij de metastasering van darmkanker.

De histologische factoren zijn niet alleen herkenbaar in de primaire tumor, maar ook in de metastasen. In **hoofdstuk 1** beschrijf ik het literatuuronderzoek naar histologische factoren in colorectale lever metastasen. Verschillende factoren blijken geassocieerd met een slechte uitkomst: lymfvat- en bloedvatinvasie, de aanwezigheid van een fibreus kapsel en het hebben van positieve resectiemarges. Uit onze studie bleek ook dat het histologisch onderzoek van colorectale levermetastasen nog in de kinderschoenen staat: goed gedefinieerde factoren, onderzocht in homogene patiëntpopulaties zijn nodig. Daarom hebben wij in **hoofdstuk 2** enkele mogelijk prognostische factoren onderzocht in een homogene populatie van 124 patiënten met een solitaire colorectale levermetastase. In de multivariabele analyse bleek intrahepatische lymfvatinvasie geassocieerd met een slechte overleving, vooral in combinatie met bloedvatinvasie. De grootte van de metastase en een interval van minder dan 12 maanden tussen resectie van de primaire tumor en de diagnose van levermetastase waren andere onafhankelijk prognostische factoren. De aanwezigheid van intrahepatische lymfvat- en bloedvatinvasie zou daarom benoemd moeten worden in pathologie verslagen van colorectale levermetastasen. Meer onderzoek is nodig om te beoordelen of adjuvante behandeling zinvol is bij patiënten met deze slechte prognostische histologische factoren.

Identificatie van mutaties in proto-oncogenen *KRAS* en *NRAS* als predictieve markers voor respons op anti-EGFR therapie heeft de patiëntselectie verbeterd. Echter, de respons op anti-EGFR therapie is ook beperkt in een groot aantal patiënten zonder mutaties in *RAS*. *RAS* mutatie analyse wordt meestal uitgevoerd op de primaire tumor omdat weefsel van de metastase niet altijd beschikbaar is. Een verschil in mutatie status tussen de primaire tumor en metastase zou een verklaring kunnen vormen voor het lage succes percentage van anti-EGFR therapie in tumoren zonder *RAS* mutatie. In **hoofdstuk 3** hebben we de concordantie van de *KRAS* mutatie status in 305 primaire colorectale tumoren en hun bijbehorende lever metastasen onderzocht. *KRAS* mutaties waren aanwezig in 35.4% van primaire tumoren. In 11 gevallen (3.6%) constateerden wij een verschil tussen primaire tumor en metastase: 5 primaire tumoren hadden een *KRAS* mutatie die werd niet gevonden in de metastase, 1 primaire tumor had geen mutatie in *KRAS* maar wel een *KRAS* mutatie in de

metastase, en in 5 gevallen hadden de primaire tumor en metastase een andere *KRAS* mutatie. De discordantie was derhalve klinisch relevant in slechts 6 patiënten (2.0%). Daarom concluderen wij dat zowel de primaire tumor als de levermetastase gebruikt kan worden voor *KRAS* mutatie analyse.

In **hoofdstuk 4** hebben we het moleculair onderzoek uitgebreid. Naast primaire tumoren met levermetastasen, hebben we ook primaire tumoren met bijbehorende longmetastasen geanalyseerd, om tevens inzicht te krijgen in de relatie tussen mutatie status en metastaseringspatroon. Naast een uitgebreider onderzoek van *KRAS*, is ook de mutatie status van de proto-oncogenen *BRAF*, *HRAS*, *NRAS* en *PIK3CA* onderzocht met behulp van next-generation sequencing met specifieke probes. Het aantal verkregen unieke 'reads' was niet altijd voldoende om betrouwbaar de aan- of afwezigheid van mutaties te detecteren in alle regio's van interesse. Gepaarde resultaten van alle vijf genen werden verkregen in 249 van de 402 gevallen (62%). Met deze unieke next-generation sequencing techniek, vonden wij een hoge concordantie in *RAS/RAF* mutatie status tussen tumoren en bijbehorende metastasen, wat inhoudt dat zowel primaire tumoren als metastasen gebruikt kunnen worden om de mutatie status te bepalen voor anti-EGFR therapie. Long metastasen hadden een hoger percentage *RAS* mutaties ten opzichte van lever metastasen (71% versus 48%). In dit onderzoek hebben we aangetoond dat next-generation sequencing met specifieke probes mogelijk is, maar op paraffine materiaal wat vaker onvoldoende resultaat geeft.

Foci van tumorcellen kunnen worden gevonden in het pericolische of perirectale vetweefsel, zonder dat er lymfklier weefsel aanwezig is. Deze foci worden tumor deposities genoemd. De etiologie van tumor deposities is onduidelijk, ze zijn momenteel in stadiering opgenomen als een afzonderlijke lymfeklier categorie (N1c). Echter, als tumor deposities een prognostisch andere waarde hebben dan lymfkliermetastasen, dan zou een specifieke sub-stadiering beter zijn. In **hoofdstuk 5** hebben we onderzocht of tumor deposities gelijk zijn aan lymfkliermetastasen met betrekking tot prognose. Wij hebben een systematisch literatuuronderzoek en meta-analyse uitgevoerd naar de prognostische waarde van tumor deposities in darmkanker. 17 artikelen met 10106 patiënten werden geïncludeerd en de incidentie van tumor deposities was 22%. Tumor deposities komen vaker voor in tumoren met lymfkliermetastasen en vaatinvasie. Tumor deposities zijn geassocieerd met een slechte ziektevrije overleving, kanker specifieke overleving en totale overleving met een hazard ratio van 2. Daarnaast hebben we in vier grote cohorten, met in totaal 4914 patiënten, het effect van tumor deposities, lymfkliermetastasen en vaatinvasie op het metastaseringspatroon onderzocht. Logistische regressie toonde dat zowel tumor deposities als lymfkliermetastasen het risico op lever, long en peritoneale metastasen verhoogt. Het risico op lever metastasen is significant verhoogd in patiënten met

zowel tumor deposities als lymfkliermetastasen vergeleken bij patiënten met alleen lymfkliermetastasen. Extramurale vaatinvasie is geassocieerd met een verhoogd risico op lever- en long metastasen, maar niet met peritoneale metastasering. Onze studie toont aan dat tumor deposities biologisch en prognostisch niet hetzelfde zijn als lymfkliermetastasen of extramurale vaatinvasie. Door tumor deposities in de N1c categorie te stoppen, gaat er waardevolle prognostische informatie verloren. Daarom zouden tumor deposities apart moeten worden opgenomen in de stadiering van darmkanker.

Om meer inzicht te krijgen in de rol van lymfklieren in metastasering op afstand hebben we in **hoofdstuk 6** het metastaseringspatroon onderzocht in een obductiestudie met 1393 patiënten met metastasen op afstand, in relatie tot de aanwezigheid van regionale lymfkliermetastasen. Peritoneale metastasering en metastasen in lymfklieren op afstand kwamen vaker voor in de groep met regionale lymfkliermetastasen (28% versus 21% en 25% versus 15%). De incidentie van lever- en longmetastasen is vergelijkbaar in beide groepen. We hebben onze bevindingen gevalideerd in een grote cohort studie op basis van 2382 patiënten van de Eindhovense kanker registratie en vonden hier vergelijkbare resultaten. De hogere incidentie van peritoneale metastasen en lymfkliermetastasen op afstand, maar de vergelijkbare incidentie van lever- en long metastasen, suggereert dat regionale lymfkliermetastasen een beperkte invloed op het metastaseringspatroon hebben. Lymfkliermetastasen moeten gezien worden als teken van een agressieve tumor, maar lijken zelf niet betrokken in het metastaseringsproces. Tumorgroei langs zenuwen, perineurale invasie (PNI), is beschreven in vele vormen van kanker. De prognostische waarde van PNI voor darmkanker is onduidelijk. In **hoofdstuk 7** onderzoeken we het effect van PNI door middel van een literatuur studie. In totaal hebben we 58 studies geïncludeerd, bestaande uit 22900 patiënten, met een incidentie van 18%. PNI komt vaker voor in rectale tumoren vergeleken met colon tumoren (22% vs. 16%) en is geassocieerd met TNM stadiering. Onze meta-analyse toont een sterke invloed van de PNI op lokaal recidief, maar ook een sterk prognostisch effect op ziektevrije overleving, kanker specifieke overleving en totale overleving. Het effect van PNI was vergelijkbaar met dat van bekende/ gevestigde prognostische factoren. Daarom adviseren wij om PNI op te nemen in de gestandaardiseerde rapportage van tumoren.

Vaatinvasie is de aanwezigheid van tumorcellen in bloedvaten. Het kan worden onderverdeeld in intramurale vaatinvasie (IMVI) en extramurale vaatinvasie (EMVI), afhankelijk van de locatie van de vaten ten opzichte van de spierwand. EMVI is een bekende onafhankelijke prognostische factor, de prognostische betekenis van IMVI is minder duidelijk. **In hoofdstuk 8** rapporteren we de impact van IMVI in darmkanker. In totaal omvat de meta-analyse 20 artikelen, bestaande uit 8078 patiënten. De

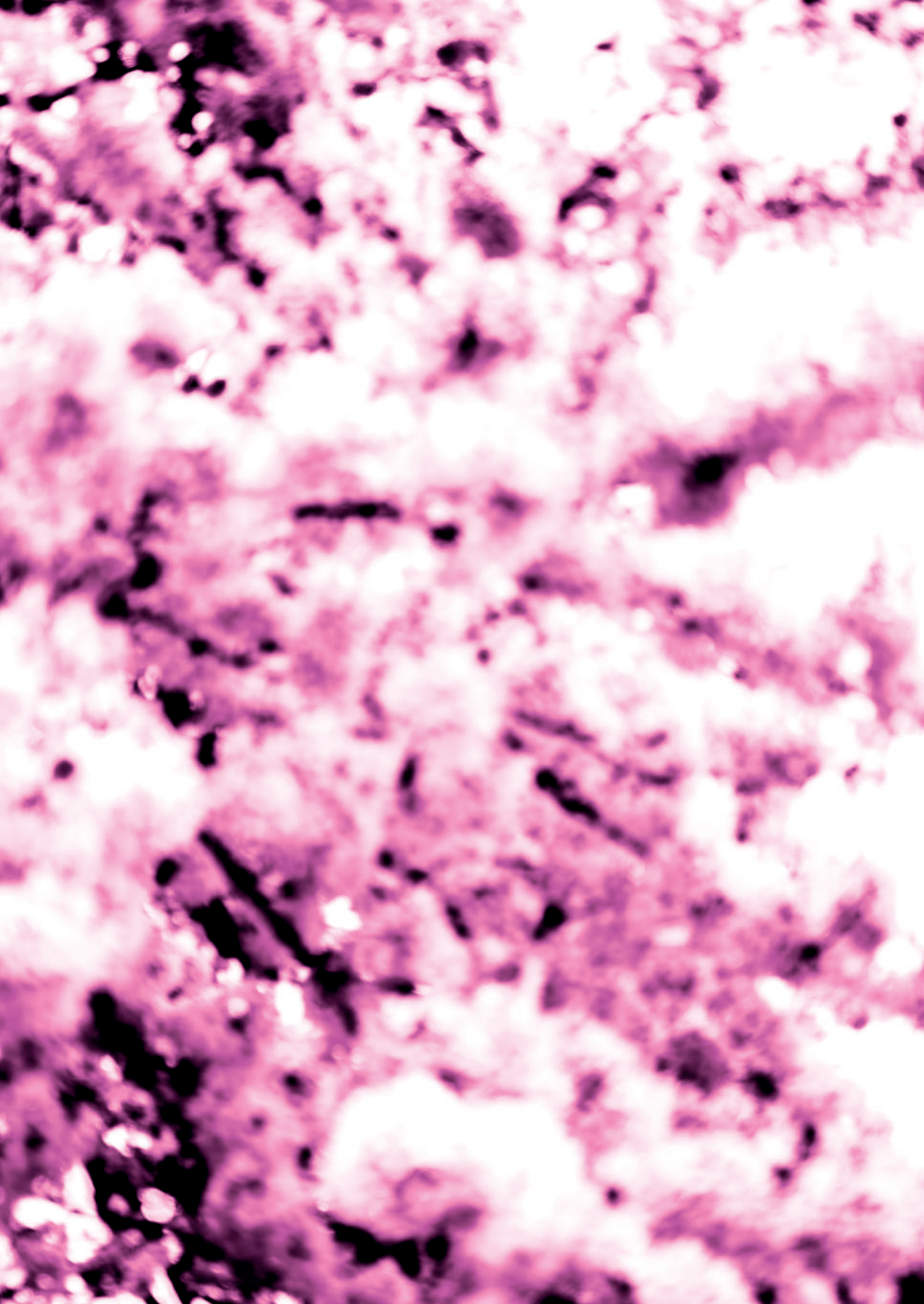
incidentie van IMVI is 12.5% en IMVI is geassocieerd met een slechtere kanker specifieke overleving. We concluderen dat, ondanks het beperkte aantal studies, er een duidelijk verband is tussen IMVI en een slechtere overleving. Er zou daarom meer aandacht voor IMVI moeten komen en een betere rapportage hiervan in pathologie verslagen.

Veel gepubliceerde histopathologische artikelen zijn retrospectief van aard en dit beperkt de kwaliteit van bewijs van deze studies. Een hoger niveau van bewijs, welke noodzakelijk is om implementatie van een factor in de dagelijkse klinische praktijk te rechtvaardigen, kan worden bereikt door systematische reviews en meta-analyses van gepubliceerde studies. Deze meta-analyse zijn echter gebaseerd op individuele studies welke sterk verschillen in kwaliteit. **In de appendix** doen wij een voorstel ter verbetering van de rapportage van retrospectieve histopathologische studies. Naleving van richtlijnen met betrekking tot rapportage zal leiden tot een kwaliteitsverbetering van histopathologische studies. Daarnaast zal het de vergelijking van resultaten tussen verschillende studies vergemakkelijken en uiteindelijk helpen bij het implementeren van nieuwe biomarkers in de dagelijkse praktijk.

Algemene conclusie

Dit proefschrift richt zich op de verschillende histologische en moleculaire factoren die mogelijk van invloed zijn op de prognose en verspreiding van darmkanker. Wij hebben aangetoond dat in colorectale levermetastasen de invasie van tumorcellen in lymfvaten geassocieerd is met een slechte overleving. Rapportage van deze factor en andere potentieel prognostische factoren in de pathologie verslagen van colorectale lever metastasen, kan leiden tot een meer geïndividualiseerde patiëntenzorg. We hebben ook gekeken naar factoren in de primaire tumor die mogelijk van invloed zijn op overleving. Onze meta-analyses hebben bewijs geleverd om perineurale invasie en intramurale vaatinvasie op te nemen in de TNM stadiering. In de TNM stadiering nemen regionale lymfklieren een centrale rol in en adjuvante behandeling is grotendeels gebaseerd op de aanwezigheid van lymfkliermetastasen. Wij twijfelen aan de rol van deze lymfkliermetastasen in metastasering van darmkanker, onder meer omdat we hebben aangetoond dat andere factoren, zoals tumor deposities en vaatinvasie, belangrijker zijn in de metastasering van darmkanker. We adviseren om tumor deposities apart te scoren, aangezien ze extra informatie geven over prognose en invloed hebben op het metastaseringspatroon. Lymfkliermetastasen moeten worden gezien als teken van gevorderde ziekte, maar zijn zelf niet betrokken bij metastasering van darmkanker.

Naast prognostische factoren zijn ook predictieve factoren belangrijk om de behandeling van patiënten met darmkanker te kunnen individualiseren. De meest bekende predictieve marker in darmkanker is de *RAS* mutatie status, waarbij voorafgaand aan anti-EGFR therapie altijd moleculaire analyse wordt verricht. Wij hebben aangetoond dat er een hoge concordantie van *RAS* mutatie status is tussen primaire tumor en metastasen. Hierdoor kan de primaire tumor gebruikt worden voor analyse van de *RAS* mutatie status. Discordantie in testresultaten vormt derhalve geen verklaring voor het falen van anti-EGFR therapie in patiënten met tumoren zonder *RAS* mutatie.



Curriculum Vitae and List of publications

Curriculum Vitae

Nikki Knijn is op 7 juli 1986 in Avenhorn geboren. In 2004 behaalde ze haar VWO diploma aan de Openbare Scholen Gemeenschap te Hoorn. Vanaf 2004 studeerde ze Geneeskunde aan de Radboud Universiteit Nijmegen. Tijdens de opleiding Geneeskunde nam ze deel aan het Honours Programma. Daarnaast kwam ze in aanraking met onderzoek en heeft ze van 2006 tot 2011 als student-assistent werkzaamheden verricht op de afdeling pathologie, ten behoeve van de CAIRO studies van de Dutch Colorectal Cancer Group. De wetenschappelijke stage binnen de opleiding Geneeskunde heeft ze verricht in Leeds, onder begeleiding van prof. Matt Seymour en prof. Phil Quirke. Na haar arts-examen in 2011 is ze in 2012 gestart met haar specialisatie tot klinisch patholoog bij het Radboudumc in Nijmegen (opleiders prof. Piet Slootweg en dr. Willeke Blokx). In 2013 heeft ze de opleiding onderbroken om verder te gaan met haar PhD traject onder supervisie van prof. Iris Nagtegaal en prof. Kees Punt, gefinancierd door middel van een persoonlijke beurs voor arts-assistenten van KWF Kankerbestrijding. In 2016 heeft ze haar opleiding tot patholoog hervat en heeft ze onder meer stage gelopen in het Canisius Wilhelmina Ziekenhuis (Nijmegen) en het Rijnstate Ziekenhuis (Arnhem). Naast haar opleiding is ze actief betrokken bij het ontwikkelen en geven van onderwijs. In 2018 heeft ze het Christine Mohrmann stipendium toegewezen gekregen. Doel van het stipendium is om vrouwelijke promovendi aan te moedigen hun wetenschappelijke loopbaan na de voltooiing van hun proefschrift voort te zetten. Nikki zal dit stipendium gebruiken voor haar verdiepingsstage aan de University of California, San Francisco (UCSF).

Nikki woont samen met Jean in Nijmegen. Samen hebben zij drie kinderen; Ezra en Senn (2013) en Liz (2016).

About the author

Nikki Knijn was born on the 7th of july 1986 in Avenhorn (the Netherlands). In 2004 she obtained her Gymnasium diploma at the Openbare Scholen Gemeenschap in Hoorn (the Netherlands). In 2004 she started her medical education at the Radboud University in Nijmegen (the Netherlands). During her medical training she preformed research at the department of Pathology on behalf of the CAIRO studies of the Dutch Colorectal Cancer Group. She performed her scientific internship at the department of Pathology in Leeds (United Kingdom), under supervision of prof. Matt Seymour and prof. Phil Quirke. After completing her medical training, Nikki started in 2012 as a trainee in Pathology at the Radboud university medical center (supervisors prof. Piet Slootweg and dr. Willeke Blokx). In 2013 she started her PhD trajectory under supervision of prof. Iris Nagtegaal and prof. Kees Punt. She received a personal oncology research grant for residents of the Dutch Cancer Society (KWF Kankerbestrijding). In 2016 Nikki continued her pathology training. In 2018 Nikki will receive the Christine Mohrman Stipendium. She will use this stipendium for her stay at the University of California, San Francisco.

Nikki lives in Nijmegen together with Jean and their three children; Ezra and Senn (2013) and Liz (2016).

List of publications

Siddiqui M, Nagtegaal I, Santiago I, **Knijn N**, Berho M, Mirnezami A, Rao S, Brown G. *Session 2: What causes liver metastases – lymph nodes or is it something else?* Colorectal Disease 2018; 20 Suppl 1:39-42.

Pagès F, Mlecnik B, Marliot F, Bindea G, Ou FS, Bifulco C, Lugli A, Zlobec I, Rau TT, Berger MD, Nagtegaal ID, Vink-Börger E, Hartmann A, Geppert C, Kolwelter J, Merkel S, Grützmann R, Van den Eynde M, Jouret-Mourin A, Kartheuser A, Léonard D, Remue C, Wang JY, Bavi P, Roehrl MHA, Ohashi PS, Nguyen LT, Han S, MacGregor HL, Hafezi-Bakhtiari S, Wouters BG, Masucci GV, Andersson EK, Zavadova E, Vocka M, Spacek J, Petruzelka L, Konopasek B, Dundr P, Skalova H, Nemejcova K, Botti G, Tatangelo F, Delrio P, Ciliberto G, Maio M, Laghi L, Grizzi F, Fredriksen T, Buttard B, Angelova M, Vasaturo A, Maby P, Church SE, Angell HK, Lafontaine L, Bruni D, El Sissy C, Haicheur N, Kirilovsky A, Berger A, Lagorce C, Meyers JP, Paustian C, Feng Z, Ballesteros-Merino C, Dijkstra J, van de Water C, van Lent-van Vliet S, **Knijn N**, Mușină AM, Scripcariu DV, Popivanova B, Xu M, Fujita T, Hazama S, Suzuki N, Nagano H, Okuno K, Torigoe T, Sato N, Furuhata T, Takemasa I, Itoh K, Patel PS, Vora HH, Shah B, Patel JB, Rajvik KN, Pandya SJ, Shukla SN, Wang Y, Zhang G, Kawakami Y, Marincola FM, Ascierto PA, Sargent DJ, Fox BA, Galon J. *International validation of the consensus immunoscore for the classification of colon cancer: a prognostic and accuracy study.* Lancet 2018; 391(10135):2128-2139.

Knijn N, van Exsel UEM, de Noo ME, Nagtegaal ID. *The value of intramural vascular invasion in colorectal cancer – a systematic review and meta-analysis.* Histopathology 2018; 72(5):721-728.

Nagtegaal ID, **Knijn N**, Hugen N, Marshall HC, Sugihara K, Tot T, Ueno H, Quirke P. *Tumor deposits in colorectal cancer: improving the value of modern staging – a systematic review and meta-analysis.* Journal of Clinical Oncology 2017; 35(10):1119-1127.

Knijn N, van Erning FN, Overbeek LI, Punt CJ, Lemmens VE, Hugen N, Nagtegaal ID. *Limited effect of lymph node status on the metastastic pattern in colorectal cancer.* Oncotarget 2016; 7(22):31699-707.

Knijn N, Mogk S, Teernestra S, Simmer F, Nagtegaal ID. *Perineural invasion is a strong prognostic factor in colorectal cancer.* American Journal of Surgical Pathology 2016; 40(1)103-12.

de Ridder JA, **Knijn N**, Wiering B, de Wilt JH, Nagtegaal ID. *Lymphatic Invasion is an Independent Adverse Prognostic Factor in Patients with Colorectal Liver Metastasis.* Annals of Surgical Oncology 2015; 22(3):638-645.

Knijn N, Simmer F, Nagtegaal ID. *Recommendations for reporting histopathology studies: a proposal.* Virchows Archiv 2015; 466(6):611-615.

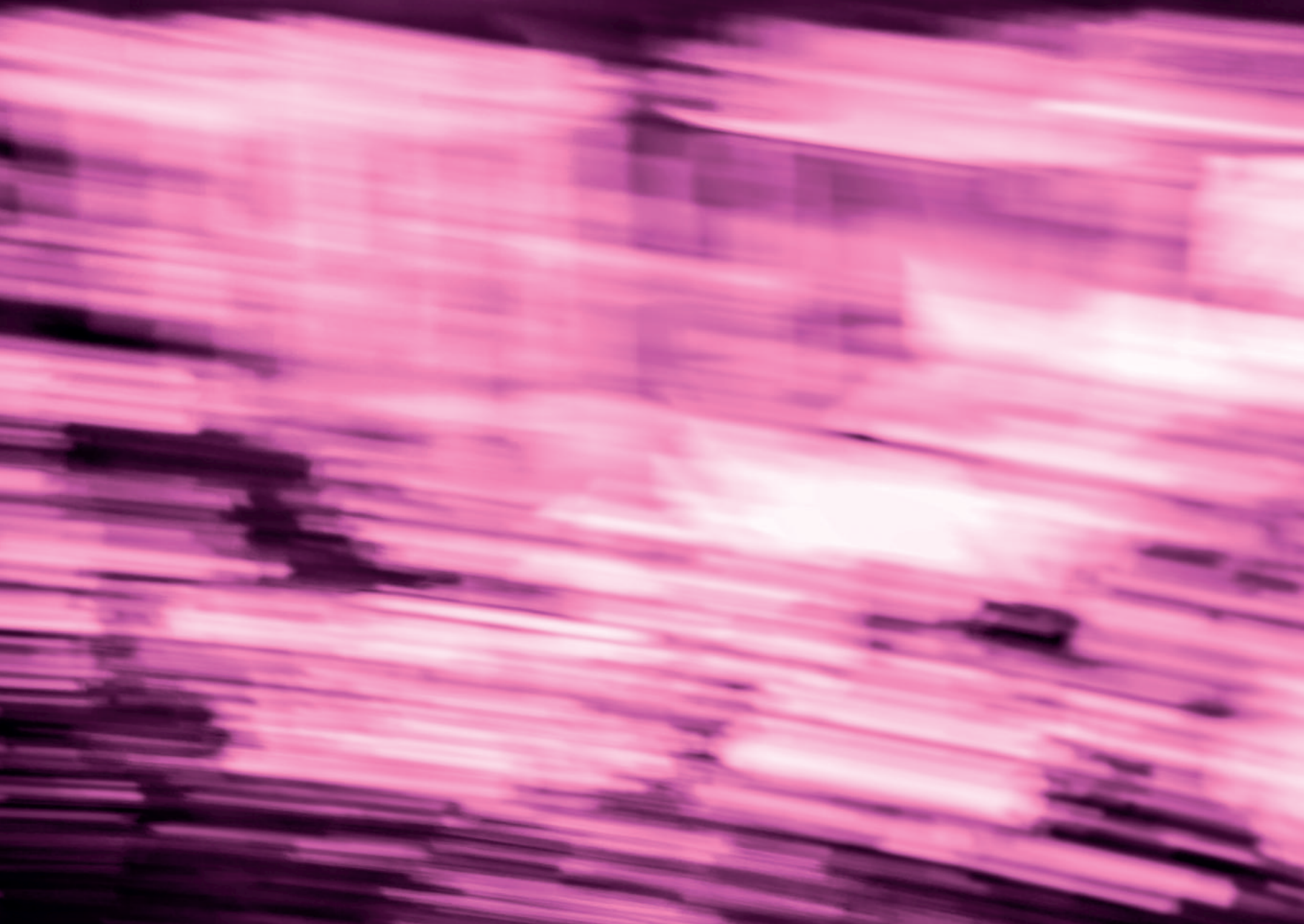
Knijn N, Nagtegaal ID. *Guidelines for reporting histopathology studies.* Journal of Clinical Pathology 2015; 68(2):173-174.

Knijn N, de Ridder JA, Punt CJ, de Wilt JH, Nagtegaal ID. *Histopathological evaluation of resected colorectal cancer liver metastases: what should be done?* Histopathology 2013;63(2):149-156. Review.

Knijn N, Mekenkamp LJM, Klomp M, Vink-Börger ME, Tol J, Teerenstra S, Meijer JW, Tebar M, Riemersma S, van Krieken JHJM, Punt CJA, Nagtegaal ID. *KRAS mutation analysis: a comparison between primary tumours and matched liver metastases in 305 colorectal cancer patients.* British Journal of Cancer 2011; 104(6):1020-1026.

Knijn N, Tol J, Koopman M, Werter MJ, Imholz AL, Valster FA, Mol L, Vincent AD, Teerenstra S, Punt CJA. *The effect of prophylactic calcium and magnesium infusions on the incidence of neurotoxicity and clinical outcome of oxaliplatin-based systemic treatment in advanced colorectal cancer patients.* European Journal of Cancer 2011; 47(3):369-374.

Knijn N, Tol J, Punt CJA. *Current issues in the targeted therapy of advanced colorectal cancer*. Discovery Medicine 2010; 9(47):328-336.



Dankwoord

Dw

Dankwoord

En dan ben je nu aangekomen bij het dankwoord. Misschien ben je hier als eerste naar toe gebladerd en heb je het voorgaande niet gelezen. Dit is ook een belangrijk (en misschien wel het meest gelezen) stuk van het proefschrift. Onderzoek doen kan alleen met hulp van anderen, die ik hieronder zal noemen.

Allereerst mijn promotoren.
Prof. dr. Nagtegaal, beste Iris, door jou ben ik in dit onderzoek en op de afdeling pathologie beland. Dus eigenlijk dank ik mijn carrière in de pathologie aan jou ;-). Jouw fijne en intensieve begeleiding in het onderzoek, is een luxe die maar weinig promovendi hebben. Door alle wekelijkse gesprekken, kwam er al snel een goede structuur en lijn in het onderzoek. Jouw enthousiasme en ideeën werken aanstekelijk en hebben ervoor gezorgd dat ik de vele projecten tot het eind toe met veel plezier en vol motivatie kon afronden.

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